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Article

Synthesis and Anti-HCV Activities of 4'-Fluoro-2'-Substituted Uridine Triphosphates and Nucleotide Prodrugs: Discovery of 4'-Fluoro-2'-C-Methyluridine 5'-Phosphoramidate Prodrug (AL-335) for the Treatment of Hepatitis C Infection

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Synthesis and Anti-HCV Activities of 4'-Fluoro-2'-Substituted Uridine Triphosphates and Nucleotide Prodrugs: Discovery of 4'-Fluoro-2'-*C*-Methyluridine 5'-Phosphoramidate Prodrug (AL-335) for the Treatment of Hepatitis C Infection

Guangyi Wang,[1] Natalia Dyatkina,[1] Marija Prhavc,[1*] Caroline Williams,[1,a] Vladimir Serebryany,[1,a] Yujian Hu,[2] Yongfei Huang,[2] Jinqiao Wan,[2,b] Xiangyang Wu,[2] Jerome Deval,[1,a] Amy Fung,[1] Zhinan Jin,[1] Hua Tan, [1,a] Kenneth Shaw,[1] Hyunsoon Kang,[1] Qingling Zhang, [1,a] Yuen Tam,[1] Antitsa Stoycheva, [1,a] Andreas Jekle, [1,a] David B. Smith, [1,a] Leonid Beigelman[1,a]

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Abstract: We report synthesis and biological evaluation of a series of 4'-fluoro-2'-*C*-substituted uridines. Triphosphates of the uridine analogues exhibited potent inhibition of HCV NS5B polymerase with IC_{50} values as low as 27 nM. In an HCV subgenomic replicon assay, the phosphoramidate prodrugs of these uridine analogues demonstrated very potent activity with EC_{50} values as low as 20 nM. A lead compound (**53**) demonstrated high levels of the NTP in vitro in primary human hepatocytes and Huh-7 cells as well as in dog liver following a single oral dose. Compound **53** (**AL-335**) was selected for clinical development where it showed promising results in Phase 1 and II trials.

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INTRODUCTION

Hepatitis C is a major liver disease caused by the hepatitis C virus (HCV). HCV can cause both acute and chronic hepatitis, ranging in severity from a mild illness lasting a few weeks to a serious, lifelong illness. Globally, an estimated 71 million people have chronic hepatitis C infection.[1] A significant number of those who are chronically infected will develop cirrhosis or liver cancer. Approximately 399,000 people die each year from hepatitis C, mostly from cirrhosis and hepatocellular carcinoma.[1] The standard of care for hepatitis C is changing rapidly. Sofosbuvir,[2] daclatasvir[3] and sofosbuvir/ledipasvir combination[4] are among the preferred regimens in the WHO guidelines. Therapy with these direct antiviral agents can achieve cure rates above 95% after a typical treatment period of 12 weeks. However, access to HCV treatment remains low though it is improving. In 2015, of the 71 million persons living with HCV infection globally, only 1.1 million of people were started on treatment.[1] The long term use of these drugs may lead to viral resistance and a higher rate of adverse effects over time. Therefore, a combination of safe and effective drugs with a shorter treatment period is highly desirable. A shorter duration of treatment is expected to increase treatment accessibility to patients infected with HCV.

Nucleoside analogues have a proven record as antiviral drugs as their phosphate metabolites may selectively inhibit viral targets, particularly viral polymerases, and effectively prevent viral replication.[5] This class of antiviral drugs typically exhibits a high barrier to viral resistance, which makes nucleotide analogues a “backbone” of direct acting antiviral combination therapies. Two types of modified nucleosides emerged as inhibitors of HCV polymerase, including 2’-methyl- and 4’-azido-nucleosides.[6] The well-known HCV drug sofosbuvir, a 2’-methyluridine analogue, is widely used in HCV therapies.[2] However, development of tri-isobutyrate ester of 4’-azidocytidine (Balaprivir) was discontinued due to safety reasons.[6] Another nucleoside drug BMS-

986094 (INX-08189), a 2'-*C*-methylguanosine prodrug, demonstrated very potent activity against HCV infection.[7a] However, the drug failed in a Phase 2 clinical trial because of its severe toxicity.[7b] Incorporation of 2'-*C*-methylguanosine into host RNA by human mitochondrial RNA polymerase (HMRP) may have been at least partially responsible for the toxicity.[8]

In our research, we explored a variety of sugar modified nucleoside analogues to identify a potent and safe nucleoside analogue for HCV infection. In this article, we report synthesis, HCV NS5B inhibition, host polymerase inhibition, and HCV replicon activity of a series of uridine analogues having 4'-fluoro-2'-*C*-substituted sugar moieties (Figure 1).[11] For a lead prodrug (**53**), in vitro and in vivo nucleoside triphosphate (NTP) formation is also presented. Compound **53** (**AL-335**) in combination with simeprevir and odalasvir was evaluated in human Phase 1 and 2 clinical trials and demonstrated promising efficacy and safety results,[12a,b,13] achieving 100% sustained viral response after 6-weeks of treatment in patients with genotype 1 HCV infection. Updated results on clinical trials will be published elsewhere.

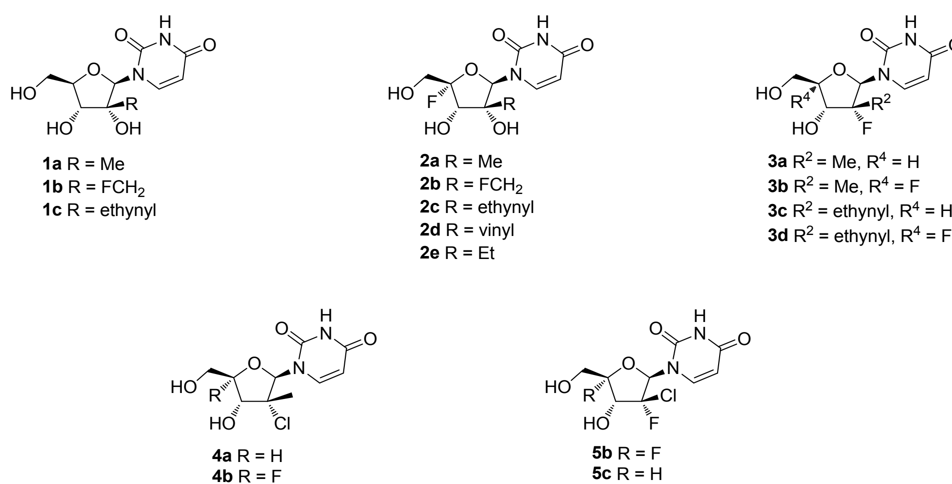

Figure 1. 2'-*C*-Substituted uridines and 4'-fluoro-2'-*C*-substituted uridines

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RESULTS AND DISCUSSION

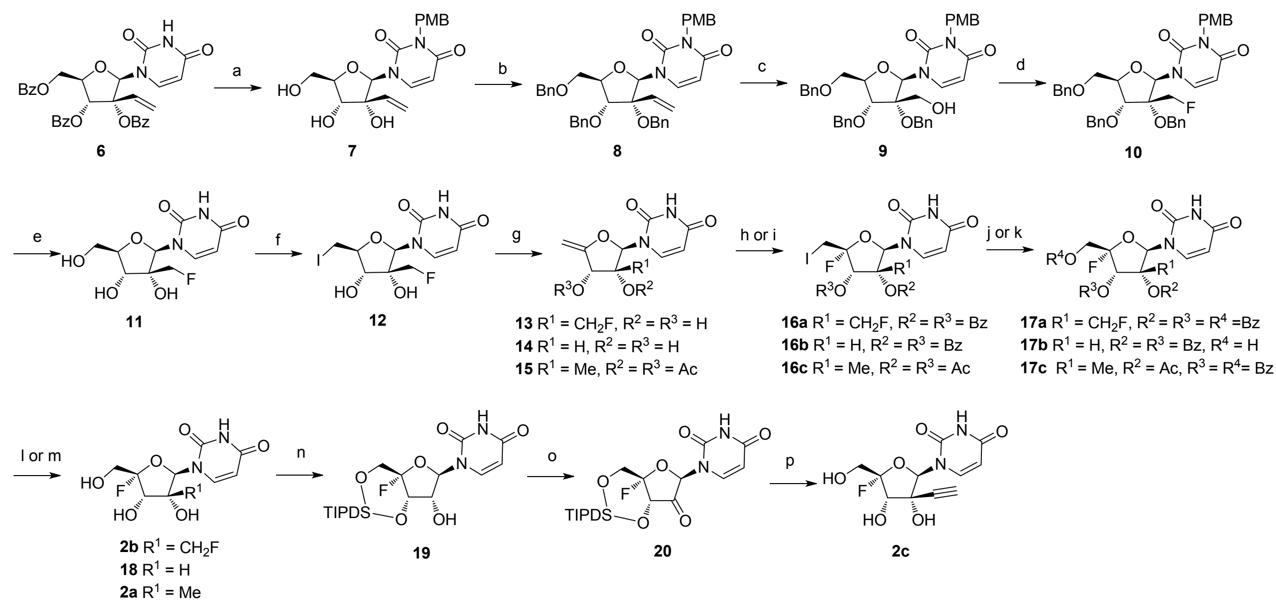
Chemistry

Synthesis of compounds **2a**, **2b** and **2c** is shown in Scheme 1. Previously we reported the synthesis of these compounds,[14a] later it was repeated by Wang et al.[14b] Synthesis of **2b** was started with the known compound **6**.[15a] The uracil base of **6** was protected with 4-methoxybenzyl and the sugar hydroxyls with benzyl. The resulting **8** was converted to the 2'-hydroxymethyl derivative **9** in good yield by oxidative cleavage and subsequent reduction. The fluorination of **9** was achieved by mesylation and treatment with TBAF. Removal of PMB and debenzylation gave, in good yield, the unprotected **11**,[15b] which was subjected to iodination, followed by elimination, to give the olefinic intermediate **13**.

In general introduction of 4'-F was achieved by utilizing the strategy initially developed by Moffat *et al.*[16] in their synthesis of nucleocidine and later utilized by others.[17] Iodofluorination of **13**, followed by benzoylation of the sugar hydroxyls yielded **16a** in moderate yield. Substitution of iodo with a benzoyloxy moiety to yield **17a** was accomplished by heating **16a** with sodium benzoate. Compound **2b** was obtained by debenzoylation of **17a** with ammonia in good yield. Compound **16b** was prepared from the known **14**,[18] by the same sequence of reactions used for **16a**. The iodo moiety of **16b** was replaced with m-chlorobenzoyloxy by treatment with mCPBA in a buffer (pH 4) to give **18** after debenzoylation. Compound **2a** was prepared from known **15**[19] in a similar manner as **18**. Compound **2c** was prepared by converting **18** to the ketone **20**, followed by Grignard reaction and subsequent desilylation. Unlike the usual Grignard reaction where alkyl is added to the α-face of the 2'-keto of a nucleoside, ethynyl was added primarily to the β-face to give the desired **2c** in good yield. NMR evidence (NOESY) for the configuration at C-2' was not

conclusive. However **2c** readily formed 2',3'-isopropylidene analogue which proved *ribo* configuration.

Scheme 1.



Reagents and conditions: a) i. PMBCl, K_2CO_3, DMF, rt, overnight; ii. NaOMe, MeOH, rt, 2.5 h, 75% (2 steps); b) BnBr, NaH, DMF, rt, overnight, 46%; c) i. OsO_4, NMMO, THF/H_2O, rt, 1 d; ii. $NaIO_4$, MeOH/H_2O/THF, rt, 2 h; iii. $NaBH_4$, rt, 30 min, 61% (3 steps); d) i. MsCl, DMAP, DCM, rt, 40 min, 90%; ii. TBAF, THF, 75 ^oC, 3 d, 27%; e) i. CAN, CH_3CN/H_2O, overnight,71%; ii. BCl_3, -70 ^oC, 2 h, 86%; f) PPh_3, I_2, pyridine, rt, overnight, 58%; g) DBU, THF/CH_3CN (2:1), 65 ^oC, 2 h, 55%; h) i. TEA-3HF, NIS, CH_3CN, rt, 30 min; ii. Bz_2O, DMAP, pyridine, 50 ^oC, overnight, 35% for **16a**, 59% (2 steps) for **16b**; i) i. Ac_2O, pyridine, rt, overnight; ii. AgF, I_2, DCM, rt, 5 h, 40% (2 steps) for **16c;** j) NaOBz, 15-crown-5, DMF, 130 ^oC, 6 h, 46% for **17a**, 41% for **17c**; k) 55% aq. Bu_4NOH, TFA, pH 4, mCPBA, DCM, rt, overnight, 68% for **17b**; l) 7 M NH_3/MeOH, rt, 5 h, 75% for **18**, 25% for **2b**; m) NH_3, rt, overnight, 84% for **2a**; n) $TIPDSCl_2$, pyridine, rt, overnight, 40%; o) IBX, CH_3CN, 80 ^oC, 3 h, 80%; p) i. ethynylmagnesium bromide, THF, rt, 1 h; ii. TBAF, THF, rt, 30 min, 39% (2 steps).

Synthesis of **2d** and **2e** is shown is Scheme 2, starting from the known **1c**.[14] The 2' and 3'-hydroxyls of **1c** were protected with cyclopentylidene and the 5'-hydroxyl was converted to the

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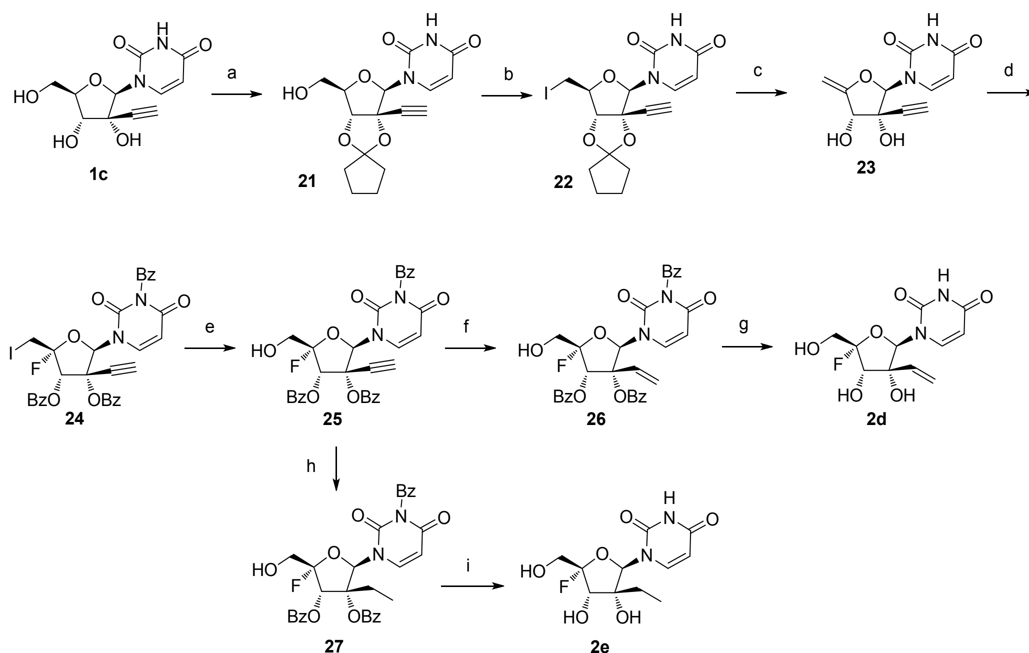
5'-iodide. The resulting **22** was subjected to the acidic hydrolysis and elimination to yield the olefin **23**. Iodofluorination of **23**, followed by benzoylation of the sugar, yielded the 4'-fluoro intermediate **24**. After hydroxydeiodination, the resulting **25** was converted to **2d** by controlled hydrogenation and subsequent hydrolysis. Similarly, **25** was converted to **2e**.

Scheme 2.



Reagents and conditions: a) TsOH, 1,1-dimethoxycyclopentane, DCE, 50 ^{o}C, overnight, 70%; b) i. TsCl, pyridine, rt, overnight; ii. NaI, acetone, reflux, overnight, 57% (2 steps); c) i. 80% AcOH, 60 ^{o}C, 2 h, 79%; ii. DBU, THF, 65 ^{o}C, 48%; d) i. TEA-3HF, NIS, CH_3CN, rt; ii. Bz_2O, DMAP, pyridine, 50 ^{o}C, 12% (2 steps); e) aq. TBAOH, , TFA, pH 4, mCPBA, rt, 24%; f) Lindlar catalyst, H2 (40 psi), EtOAc, hexanes, rt, 1.5 h; g) 7 M, NH_3 in MeOH, rt, 35% (2 steps); h) Pd/C, H_2, MeOH, rt, 1.5 h, i) 7 M NH_3 in MeOH, rt, 21% (2 steps).

Synthesis of **4a**[20,30] started with **28**[21], which was converted with tetrabutylammonium chloride at elevated temperature to the corresponding chloride via SN2 mechanism. Acidic hydrolysis followed by benzoylation afforded **30** in good yield. Conversion of **30** to **31** was achieved by

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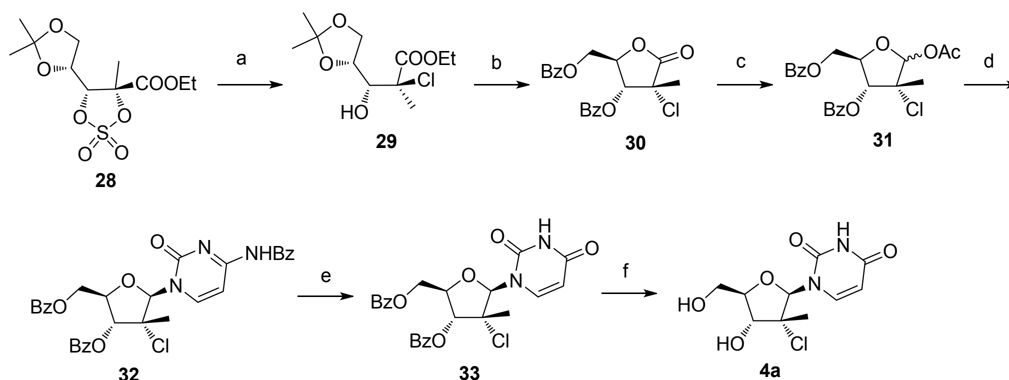
reduction and subsequent acetylation. Condensation of anomeric mixture of **31** with silylated cytosine under Vorbrueggen condition gave the cytidine **32**. Only β-cytidine was isolated and was converted to the uridine **4a** by heating with acetic acid, followed by debenzoylation and was identical to the reported 2'-chloro-2'-methyluridine.[20]

Scheme 3.



Reagents and conditions: a) i. Bu_4NCl, dioxane/H_2O, reflux, overnight; ii. $(MeO)_2CMe_2$, HCl, rt, 3 h, b) i. HCl, EtOH, rt, 48 h; ii. BzCl, pyridine, rt, 45 min, 49% (4 steps); c) i. Li(*t*-Bu)$_3$AlH, THF, -60 °C, 3 h; ii. Ac_2O, DMAP, THF, 0-5 °C, 1 h, 79% (2 steps); d) i. N-Bz-cytosine, $(NH_4)_2SO_4$, HMDS, reflux, 4 h; ii. $SnCl_4$, 65 °C, PhCl, overnight, 45%; e) AcOH/H_2O (9:1), 110 °C, overnight, 89%; f. 10 M NH_3 in MeOH, rt, overnight, 63%.

Reported synthesis of **5a**[22] via Vorbrueggen condensation using 1-*O*-acetyl sugar, 1-*O*-mesyl sugar, or **35** under various conditions yielded predominantly the α-nucleoside with low yield of desired β-isomer. In search of new synthetic route for **5a**, we found that the 1-amino sugar **37** was relatively stable, which could be used to build the uracil base on the sugar. In this way, α/β-mixture (roughly 1:1) of **5a** was prepared from known **34**,[23] as shown in Scheme 4.

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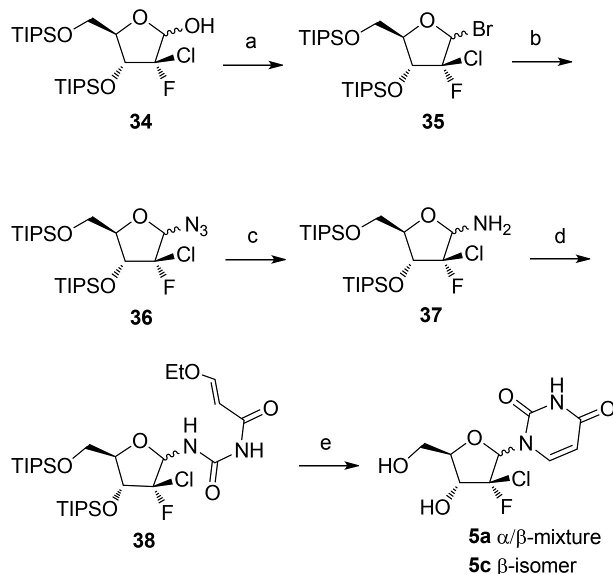
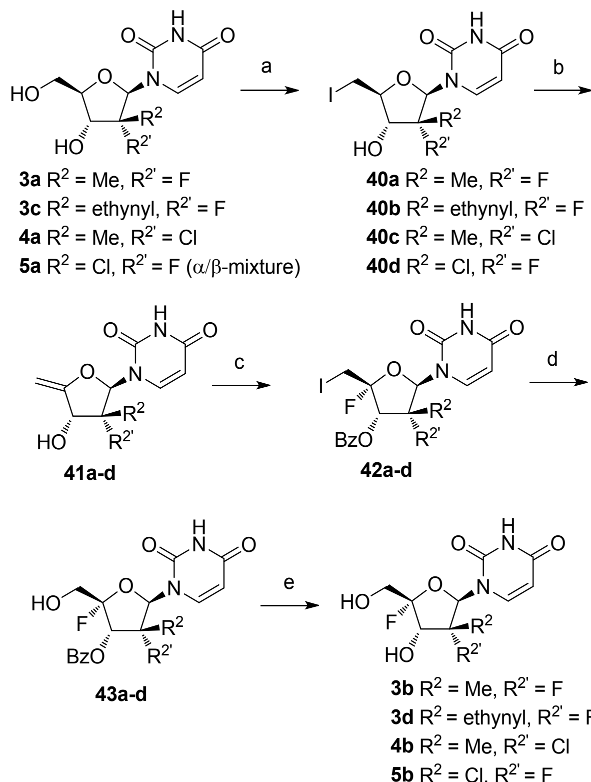
Scheme 4.



Reagents and conditions: a) CBr_4, Ph_3P, DCM, rt, 1 h, 92%; b) NaN_3, DMF, 50 °C, overnight, 89%; c) 20% $Pd(OH)_2/C$, H_2, EtOAc, rt, 2 h, 98%; d) (*E*)-3-ethoxyacryloyl isocyanate, toluene, DCM, rt, 2 h, 94%, e) i. HCl, $EtOH/H_2O$, 85 °C, 10 h; ii. NH_4F, MeOH, 70 °C, 5 h, 74% (2 steps).

Compounds **3b**, **3d**, **4b** and **5b** were synthesized from **3a**,[24] **3c**,[25] **4a,** and **5a**, respectively, by procedures similar to those described in Scheme 1. Reaction conditions are shown in the Scheme 5.

Scheme 5.



Reagents and conditions: a) PPh_3, I_2, pyridine, THF, rt, overnight, 51% for **40a**, 77% for **40b**, 85% for **40**c, 27% for **40d**; b) DBU, THF, 50 ^{o}C, overnight, 60% for **41a**, 69% for **41b**, 45% for **41c**, 78% for **41d**; c) i. TEA-3HF, NIS, CH_3CN, rt, 7 h; ii. BzCl, pyridine, rt, 5 h, 76% (2 steps) for **42a**, 37% for **42b**, 32% for **42c**, 39% for **42d**; d) Bu_4NOH, TFA, pH 4, mCPBA, DCM, rt, 5 h, 55% for **43a**, 78% for **43b**, 41% for **43c**, 57% for **43d**; e) 7 M NH_3 in MeOH, rt, 5 h, 66% for **3b**, 59% for **3d**, 53% for **4b**, 81% for **5b**.

All nucleosides in Figure 1 were converted to their 5'-triphosphates for testing in viral and human polymerase assays. For testing in cell-based assays, most of the nucleosides in Figure 1 were

converted to their 5'-phosphoramidate prodrugs (**44-52**) as shown in Table 3. For the nucleoside **2a**, several 5'-phosphoramidate prodrugs (**53-58**) and 5'-bis-phosphoramidate prodrugs (**59-60**) that have either a different alkyl ester of the alanine or a different substitutions on the phosphorus were prepared, as shown in Table 4. Among them is a pair of *P*-isomeric prodrugs **53** and **54**, which were separated from **44** by HPLC. Chemical preparation of the triphosphates and the prodrugs is described in the experimental section.

X-Ray crystal structure of compound 53.

Absolute stereochemisty of compound **53** (AL-335) was assigned by X-ray crystallography with resolution 0.83 Å. Confirmed chiralities of stereocenters are as follows: P1 is ***(S)***; C2 is ***(S)***; C3 is ***(S)***; C4 is ***(R)***; C5 is ***(R)***; C18 is ***(S)***. Atom numbers of chiral centers are shown in Figure 2. Clearly, compound **53** is the Sp isomer.

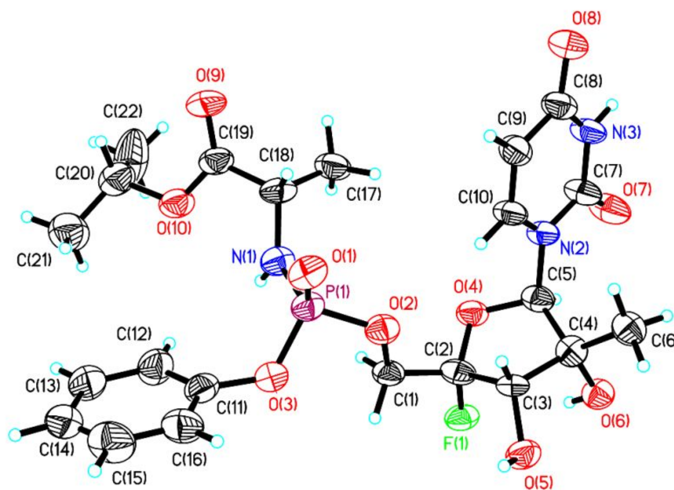


Figure 2. X-ray crystal structure of compound **53**

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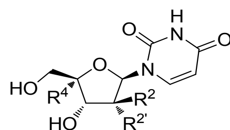
Inhibition of HCV NS5B polymerases

4'-Fluoro-2'-*C*-substituted uridine triphosphates were tested in HCV NS5B polymerase assay,[26] along with their 4'-H triphosphate analogues for comparison. As shown in Table 1, all NTPs except for **2d-TP** and **2e-TP** are potent inhibitors of HCV NS5B with IC_{50} values ranging from 0.027 to 0.86 μM. Weak inhibition by **2d-TP** and **2e-TP** could be caused by the unfavorable shape of 2'-*C*-vinyl and 2'-*C*-ethyl since **1c-TP**, **2c-TP**, **3c-TP** and **3d-TP** which have 2'-*C*-ethynyl are potent inhibitors. It is noticible that **1c-TP** and **2c-TP** which have 2'-OH are approximately 10 fold more potent than the corresponding **3c-TP** and **3d-TP** which have 2'-F. **1b-TP** and **2b-TP** which have 2'-*C*-fluoromethyl are equipotent as **1a-TP** and **2a-TP** which have 2'-*C*-methyl, implicating that the electron withdrawing nature of the fluorine did not exert a significant effect on the inhibition. Compound **4a-TP** which has 2'-α-chloro-2'-*C*-methyl exhibited very potent inhibition with an IC_{50} value of 0.062 μM, four fold more active than its closely related analogue **3a-TP,** although Cl has a larger size than F. **5c-TP** containing 2'-β-Cl-2'-α-F has an IC_{50} value of 0.30 μM, equipotent to **3a-TP**. Replacement of methyl with chlorine at the 2' position appears well tolerated although their inductive properties and electron densities are quite different. When fluorine is introduced at 4' position of the sugar, **2a-TP**, **3b-TP**, **3d-TP**, **4b-TP** and **5b-TP** have basically equipotent inhbitory effets on NS5B as the corresponding 4'-H NTPs, **1a-TP**, **3a-TP**, **3c-TP**, **4a-TP** and **5c-TP**. The 4'-F NTPs such as **2a-TP** and **4b-TP** showed very desirable inhibtion of NS5B with IC_{50} values of 0.14 and 0.11 μM, respectively. A preliminary conclusion is that introduction of fluorine at the 4' position does not alter inhibitory properties of NS5B dramatically and can lead to more potent NTPs.

Table 1. Inhibition of HCV polymerase NS5B by uridine triphosphates[a,b]

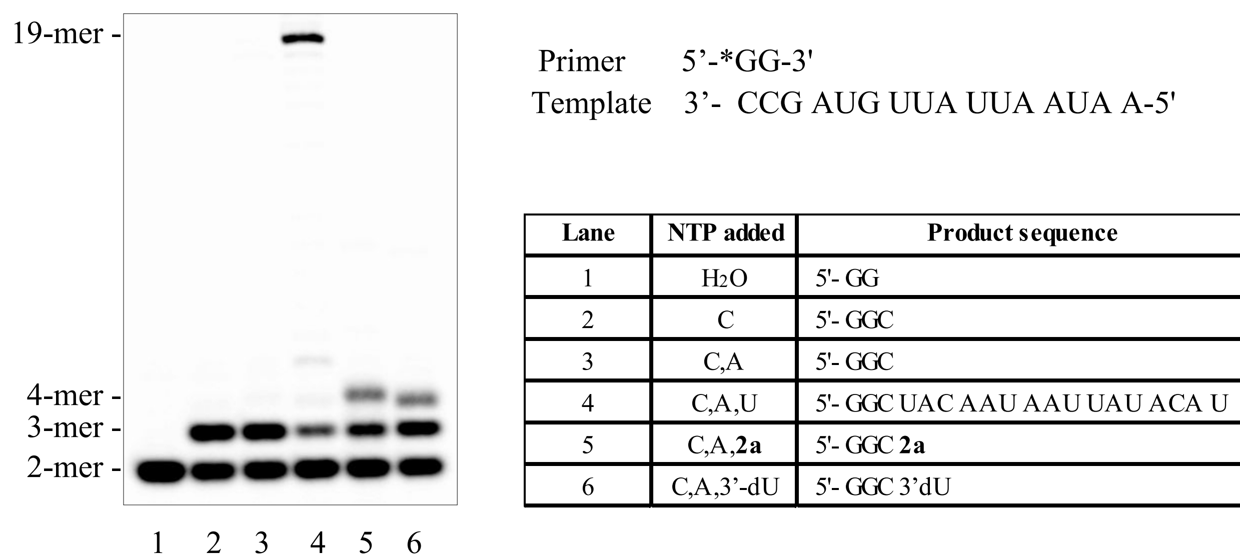


NTP	R^2	$R^{2'}$	R^4	IC_{50}, μM
1a-TP[30]	Me	OH	H	0.26
1b-TP	FCH_2-	OH	H	0.28
1c-TP	ethynyl	OH	H	0.027
2a-TP	Me	OH	F	0.14
2b-TP	FCH_2-	OH	F	0.15
2c-TP	ethynyl	OH	F	0.039
2d-TP	vinyl	OH	F	6.4
2e-TP	Et	OH	F	84
3a-TP[30]	Me	F	H	0.27
3b-TP	Me	F	F	0.86
3c-TP	ethynyl	F	H	0.33
3d-TP	ethynyl	F	F	0.34
4a-TP[30]	Me	Cl	H	0.062
4b-TP	Me	Cl	F	0.11
5b-TP	Cl	F	F	0.19
5c-TP	Cl	F	H	0.30

[a] IC_{50} indicates a concentration at which the activity of HCV polymerase NS5B is inhibited by 50%.

[b] Each IC_{50} value is an average of $n \geq 2$ determinations.

As shown in Table 1, several 4’-F NTPs are highly inhibitory to HCV NS5B polymerase. To unravel their mechanism of action, **2a-TP** was tested in a chain termination assay.[27] As shown in Figure 3, the dimer GG in the lane 2 and 3 could elongate to the trimer GGC in the presence of CTP or CTP and ATP. After adding UTP, the GG dimer could elongate to a 19-mer (lane 4). However, the elongation stopped after **2a-TP**, as a replacement of UTP, was incorporated into the sequence (lane 5). A similar termination was observed when UTP was replaced by 3’-dUTP. The results indicate that **2a** on the elongating sequence acts as an effective chain teminator.



Lane	NTP added	Product sequence
1	H_2O	5'- GG
2	C	5'- GGC
3	C,A	5'- GGC
4	C,A,U	5'- GGC UAC AAU AAU UAU ACA U
5	C,A,**2a**	5'- GGC **2a**
6	C,A,3’-dU	5'- GGC 3’dU

Figure 3. Chain termination of HCV polymerase NS5B mediated RNA Synthesis by **2a-TP**.

Inhibition of human polymerases

To assess the selectivity between viral and human polymerase inhibitions, selective NTPs from Table 1 were tested for inhibition of human DNA polymerases α, β, and γ as well as human

RNA polymerase II.[10] The results are listed in Table 2. The known 2'-fluoro-2'-*C*-methyl-UTP (**3a-TP**) and **5c-TP** as well as novel **5b-TP** showed moderate inhibition of pol-α (58-76% inhibition at 100 μM) while other NTPs did not or only weakly inhibited pol-α. The moderate inhibition by **3a-TP**, **5c-TP**, and **5b-TP** may have resulted from the replacement of 2'-OH by 2'-F as all the three nucleotides have 2'-fluoro on the α-face, which might have moderately reduced the discrimination by pol-α. All the NTPs tested had little inhibition on pol-β and pol-γ. All the NTPs tested showed IC_{50} values of >100 μM in the polymerase inhibititon assay against human RNA pol II. In an assay to investigate the nucleotide incorporation by HMRP (Figure 4), only the known 2'-*C*-methyl-UTP (**1a-TP**) and 2'-*C*-chloro-UTP (**4a-TP**) showed weak, but appreciable incorporation while all the other NTPs tested including **2a-TP** and **4b-TP** showed basically no incorporation. Overall, the results indicate that the 4'-F NTPs as well as the 4'-H NTPs are not effective inhibitors of human polymerases. In certain cases, 4'-F NTPs seem more selective. Thus, **2a-TP** was basicallly not incorporated into RNA by HMRP while **1a-TP** was appreciably incorporated. Based on the promising viral and human polymerase inhibition results, the nucleotide prodrugs of the 4'-F nucleosides together with some 4'-H analogues were evaluated in cell based assays against the HCV subgenomic replicon.

Table 2. Inhibition of human polymerases by nucleoside triphosphates

NTP	hPol-α % inhibition @ 100 μM	hPol-β % inhibition @ 100 μM	hPol-γ % inhibition @ 100 μM	hRNA pol II IC_{50}, μM
1a-TP	22.4	-0.0080	14.6	>100
2a-TP	16.1	12.6	9.6	>100
3a-TP	58.2	14.0	9.2	>100

3b-TP	40.9	15.2	-10.0	>100
4a-TP	34.5	22.6	9.5	>100
4b-TP	19.7	24.3	18.4	>100
5c-TP	70.8	24.7	15.7	>100
5b-TP	75.8	18.1	7.9	ND

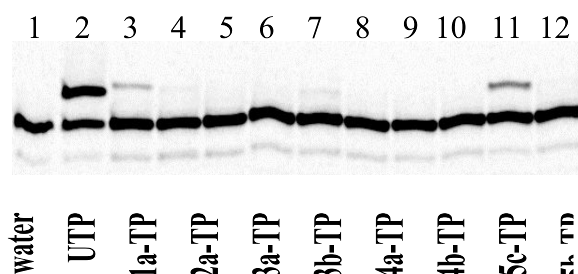


Figure 4. Nucleotide incorporation by human mitochondrial RNA polymerase

HCV replicon activity

Selective nucleosides were converted to their phosphoramidate prodrugs **44-52** and tested in an HCV subgenomic replicon system.[28] As shown in Table 3, the inhibition of HCV replicon by these phosphoramidate prodrugs correlates well with the inhibitory effects of the corresponding NTPs in the HCV polymerase assay. Prodrugs of **3b** and **4b** that have F at the 4' position have comparable, potent replicon activity (**49** and **51**: 0.31 and 0.10 μM, respectively) as prodrugs of **3a**, and **4a** that have H at the 4' position (**48** and **50**: 0.22 and 0.12 μM, respectively). As expected from HCV polymerase inhibition data, the prodrugs **46** and **47** are only weakly active. The other three 4'-F urdine prodrugs **44**, **45** and **52** are highly potent with EC_{50} values ranging from 0.045 to

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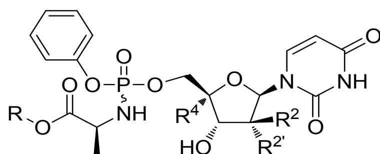
0.11 μM. Again, the 4'-fluorouridine analogues proved to be highly potent in the cell-based HCV assay.

Table 3. Inhibition of the subgenomic HCV replicon (genotype 1b) by the prodrugs of 2′-*C*-substituted uridines and 4'-fluoro-2'-*C*-substituted uridines



Prodrug (of nucleoside)	R	R^2	$R^{2'}$	R^4	EC_{50} μM	CC_{50} μM
44 (of **2a**)	i-Pr	methyl	OH	F	0.11	>100
45 (of **2c**)	c-hexyl	ethynyl	OH	F	0.07	>100
46 (of **2d**)	i-Pr	vinyl	OH	F	8.3	>100
47 (of **2e**)	i-Pr	ethyl	OH	F	43	>100
48 (of **3a**)	i-Pr	methyl	F	H	0.22	>100
49 (of **3b**)	i-Pr	methyl	F	F	0.31	>100
50 (of **4a**)	i-Pr	methyl	Cl	H	0.12	>100
51 (of **4b**)	i-Pr	methyl	Cl	F	0.10	>100
52 (of **5b**)	i-Pr	Cl	F	F	0.045	>100

EC_{50} indicates a concentration at which HCV replicon is inhibited by 50%. Each EC50 value is an average of ≥2 independent determinations. CC_{50} indicates a concentration at a which 50% of the cells died.

The nucleoside **2a** was selected for prodrug development as shown in Table 4. The two *P*-isomers of the prodrug **44** were separated by HPLC to give **53** (Sp) and **54** (Rp). The Sp-isomer **53** (EC_{50} of 0.07 μM) is 13 fold more active than Rp-isomer **54** in the HCV replicon assay. The prodrugs **55** and **56** where isopropyl was replaced by cyclohexyl and neopentyl, respectively, are more potent than **44** in the assay, with IC_{50} of 0.04 μM for both prodrugs. Likewise, when Ph was replaced by naphthyl and *p*-chlorophenyl, the resulting prodrugs **57** and **58** are also very potent,

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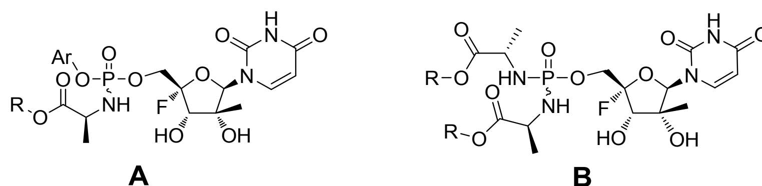
with EC_{50} of 0.02 and 0.08 μM, respectively. However, the bis-phosphoramidate prodrugs **59** and **60** are only moderately active. None of the prodrugs showed appreciable cytotoxicity in Huh-7 cells. To assess potential cytotoxicity the prodrug **53** was tested in multiple cell lines for 8 days. As shown in Table 5, the compound did not show any significant cytotoxicity and CC_{50} values are >96 μM for all 6 cell lines, including the highly sensitive U937 and MT-4 cell lines. In contrast, INX-08189, a phosphoramidate prodrug of 2'-*C*-methyl-6-*O*-methylguanosine, showed potent inhibition on several cell lines.

Table 4. HCV replicon activity of 2'-methyluridine 5'-phosphoramidate prodrugs and 4'-fluoro-2'-methyluridine 5'-phosphoramidate prodrugs



Prodrugs of **2a**	P-chirality	Scaffold	Ar	R	EC_{50}, μM	CC_{50}, μM
44 (53+54)	achiral	A	>100	i-Pr	0.11	>100
53	Chiral, Sp	A	Ph	i-Pr	0.07	>99
54	Chiral, Rp	A	Ph	i-Pr	0.94	>100.00
55	achiral	A	Ph	c-hexyl	0.04	84
56	achiral	A	Ph	neopentyl	0.04	>100.00
57	achiral	A	Naph	i-Pr	0.02	>85
58	achiral	A	p-ClPh	i-Pr	0.08	>100.00
59	NA	B	--	Et	7.5	>100.00
60	NA	B	--	i-Pr	6.6	>100.00

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Table 5. Eight-day cytotoxicity (CC_{50}, μM) of compound 53 in six cell lines

Cell Line	Huh-7	HepG2	A549	HeLa	U937	MT-4
53	96.1	>100	>100	>100	>100	>100
INX-08189	0.35	1.5	7.8	13.4	0.17	0.96

Based on its potent activity in the HCV GT-1b replicon and favorable cytotoxicity profile, the phosphoramidate prodrug **53** was further tested for inhibition of other HCV genotypes. Table 6 shows that the compound is highly potent in all genotypes tested (GT1b, GT1a, GT2b, GT3a and GT4a) with EC_{50} values ranging from 0.04 to 0.06 μM.

Table 6. HCV pan-genotypic replicon activity of compound 53

	GT1b WT	GT1a	GT2b	GT3a	GT4a
EC_{50} (μM)	0.04 ± 0.02	0.06 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.06 ± 0.01

Compound **53** is more active than the known compound sofosbuvir (0.07 μM vs 0.22 μM) in the HCV replicon assay and its NTP (**2a-TP**) is slighly more potent (0.14 μM vs 0.27 μM) than the TP of sofosbuvir (**3a-TP**). Compound **53** also demonstrated a very good in vitro safety profile as shown by the human polymerase inhibition data and 8-day cytotoxicity data. With all these favorable properties, the compound **53** was selected for further evaluation.

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In vitro and in vivo NTP formation

NTP levels in the target tissue are important to in vivo efficacy of an antiviral nucleoside polymerase inhibitor. In vitro NTP formation in appropriate cell lines may provide the first assessment for predicting the in vivo NTP level. For compound **53**, we measured the in vitro NTP levels in two cell types, primary human hepatocytes and Huh-7 cells. As shown in Table 7, high levels of NTP were formed in human hepatocytes and Huh-7 cells following an incubation of **53** at 50 μM extracellular concentration for 24 hours. In human hepatocytes, the equilibrium between NTP formation and degradation was typically reached between 6 and 24 hours (Figure 5). **2a-TP** intracellular concentrations increased approximately dose proportionally in the dose range of 0.3 to 30 μM and greater than dose proportionally from 30 to 50 μM of **53** incubation concentrations (Figure 6). Following a 24-hr incubation of **44** (~1:1 mixture of **53** and **54**) in human hepatocytes, high concentrations of NTP were formed. Once formed and after the removal of **44** from the incubation media, the NTP levels were maintained for 6 hours before degradation took place (Figure 7). The mean intracellular half-life of the NTP was thus estimated as 30.1 ± 6.3 hours (n = 3) in human hepatocytes.

In vivo NTP formation was evaluated in beagle dogs following a single oral administration of **53** at 9.87 mg/kg (5 mg/kg parent nucleoside equivalent dose). As shown in Table 7, liver NTP concentrations were 23190, 35222, and 29991 nM at 4, 12, and 24 hours post dose, respectively.

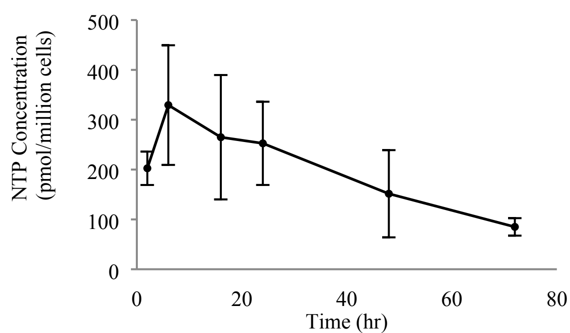

Figure 5. Time course of intracellular NTP in primary human hepatocytes following incubation with **53** at 10 µM for 2 to 72 hours

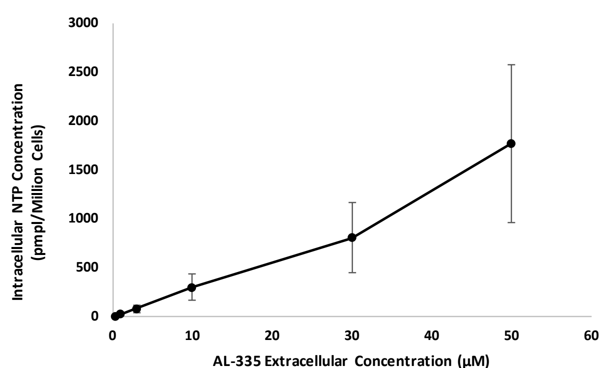

Figure 6. Intracellular NTP concentration-extracellular **53** concentration response in primary human hepatocytes[a]

[a] Data were collected from hepatocytes from three different human donors and express as mean ± SD. Each experiment was conducted with 24 hours of incubation of **53** with hepatocytes.

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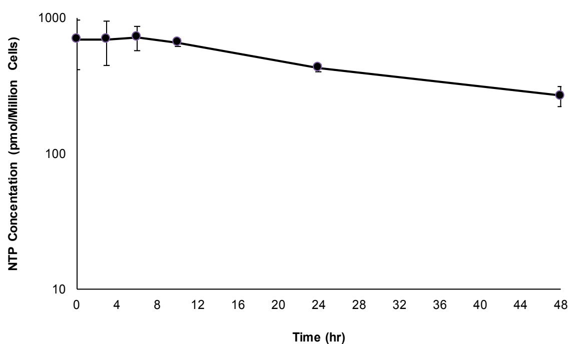

Figure 7. Intracellular NTP concentration-time profile in primary human hepatocytes following an initial 24-hour incubation with **44** at 50 μM.

Table 7. NTP formation in vitro and in vivo

Compound	NTP (pmol/Million Cells)[a]		Dog Liver NTP (nM)[b]		
	Human Hepatocytes	Huh-7	4 hr	12 hr	24 hr
53	3540 ± 3133	5695 ± 1793	23190	35222 ± 10478	29991 ± 11873

[a] Results were obtained after incubation at 50 μM of the compound for 24 hours.

[b] Results were obtained following oral administration of compound **53** at 9.87 mg/kg to dogs, N=2 at 4 hr and N=3 at 12 and 24 hr.

CONCLUSSIONS

we have synthesized a series of novel uridine analogues with 4'-fluor-2'-substituted sugar moieties. Several of the 4'-fluorouridine 5'-triphosphates demonstrated potent inhibition in the HCV polymerase NS5B assay with IC_{50} values as low as 27 nM and showed little inhibition of human DNA and RNA polymerases. The 5'-triphosphate of **2a** was confirmed to be a chain

terminator in a chain elongation assay using the HCV NS5B polymerase. Phosphoramidate prodrugs of these nucleosides exhibited very potent HCV subgenomic replicon activity with EC_{50} values as low as 20 nM. Compared with the well-known nucleoside **3a** (parent nucleoside of sofosbuvir), some of the 4'-fluoro-2'-substituted uridines demonstrated equipotent or more potent in vitro activities as shown by their 5'-triphosphate and prodrugs. All the prodrugs tested exhibited little cytotoxicity in a 3-day cytoxcity assay. A lead compound **53** demonstrated an excellent safety profile in an 8-day cytotoxicity assay using six cell lines with all CC_{50} values >96 μM. Compound **53** was also highly potent in all the HCV replicon of 4 genotypes (GT-1b, 1a, 2b, 3a, 4a). Compound **53** showed high levels of TP of its parent nucleoside (**2a-TP**) in both in vitro and in vivo studies, and the NTP has a half-life of approximately 30 hours. The high levels of the NTP in vivo and long half-life indicate that a once daily therapy may be appropriate. Due to its excellent in vitro and vivo properties, compound **53** (AL-335) was advanced into clinical development where promising results were observed in human Phase 1 and 2 clinical trials.[13]

EXPERIMENTAL SECTION

All commercially obtained solvents and reagents were used as received. All solvents used for chemical reactions were anhydrous grade, unless specifically indicated. Structures of the target compounds in this work were assigned by use of NMR and MS spectroscopy. The purities of all non-salt compounds were >95% as determined on an Agilent 1200 HPLC, XTerra 3.5μm 4.6x150mm MS C18 column, using 0.04 (v/v) TFA in water and 0.02 (v/v) TFA in acetonitrile as mobile phase. The purities of all nucleotides were >95%, determined on an Agilent 1100 HPLC, 50 mM TEAA in water and 50 mM TEAA in acetonitrile as mobile phase. ^{1}H-, ^{19}F and ^{13}C-NMR

spectra were recorded on a Bruker Advance III (400 MHz) or a Varian 400MR (400 MHz) NMR spectrometer. Chemical shifts are reported in parts per million (ppm, δ) using residual solvent line as an internal reference. Splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). Coupling constants (J) are reported in herz (Hz). Mass spectrometric analyses for nucleosides were performed on an Agilent 1200 HPLC with Agilent 6110/6140/1956C MSD mass spectrometer using ESI as ionization, Phenomenex Luna C18 5µm 5.0x20mm column; mobile phase: 0.04%(v/v) TFA in water and 0.02%(v/v) TFA in acetonitrile, 40 ^{o}C, flow rate 0.4 mL/min. Mass spectrometric analyses for nucleotides were performed on an Agilent 1100 HPLC with API 2000 LC-MS/MS System using ESI as ionization, Synergi 75x2.0 mm, 4µm Hydro-RP80Å column, 50 mM TEAA in water and 50 mM in acetonitrile, flow rate 0.4 mL/min. Work-up procedures for most of chemical reactions are the same or similar, therefore, unless specifically indicated, the work-up refers to the following procedure: the reaction mixture at 0 ^{o}C is quenched with water, diluted with EtOAc or dichloromethane, washed with 5% sodium bicarbonate and then with brine, dried over anhydrous sodium sulfate, filtered, and concentrated to dryness. Unless necessary, the word "work-up" will not be mentioned for a reaction if it follows the procedure described above. Purification on silica gel refers to a flash chromatography on a silica gel column.

2'-*C*-Hydroxymethyl-2',3',5'-tri-*O*-benzyl-*N*^{3}-(4-methoxybenzyl)uridine (9). To a solution of benzoylated nucleoside **6**[14] (50 g, 86 mmol) in DMF at 0 ^{o}C were added PMBCl (16 g, 0.1 mol) and K_2CO_3 (17.8 g, 0.13 mol). After stiring at rt for 12 h, the reaction mixture was quenched with water (100 mL), and extracted with EtOAc (3x200 mL). The organic phase was evaporated to solid residue which was dissolved in MeOH/DCM (4/1, v/v, 200 mL). The resulting solution was treated with NaOMe (16.8 g, 0.31 mol), stirred at rt for 2.5 h, and then quenched with dry ice. Usual work-

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up followed by purification on silica gel column with 1% MeOH in DCM yielded nucleoside **7** as yellow foam (25 g, 75%). MS, m/z 391.1 $[M+1]^+$. To a solution of **7** (25.5 g, 65 mmol) in DMF at 0 °C was added slowly NaH (60%, 10.5 g, 260 mmol) and the resulting mixture was stirred for 30 min. Then BnBr (36.3 g, 0.21 mol) was added and the reaction mixture was stirred at rt for 12 h. Reaction was quenched with saturated NH_4Cl (aq.). Usual work-up and purification on silica gel column using 10% EtOAc in hexanes yielded benzylated nucleoside **8** (20 g, 46%) as a white solid. MS, m/z 661.2 $[M+1]^+$. To a solution of **8** (20 g, 30 mmol) and NMMO (7 g, 60 mmol) in THF/H_2O (5/1) (100 mL) was added OsO_4 (2.6 g, 10 mmol). The resulting mixture was kept for 24 h at rt and quenched with saturated aq. $Na_2S_2O_3$. After usual work-up, the crude diol was dissolved in MeOH/H_2O/THF (170 mL/30 mL/50 mL), followed by addition of $NaIO_4$ (9.6 g, 45 mmol), and the resulting mixture was stirred at rt for 2 h. Reaction mixture was filtered and $NaBH_4$ (1.8 g, 48 mmol) was added portionwise to the filtrate at 0 °C. The resulting mixture was stirred at rt for 30 min. Ususal work-up and chromatography on silica gel column using 20% EtOAc in hexanes gave 12 g of **9** (61%). MS, m/z 665.2 $(M + 1)^+$.

2'-*C*-Fluoromethyluridine (11). To a solution of hydroxymethyl nucleoside **9** (14 g, 21 mmol) and DMAP (5.1 g, 42 mmol) in DCM (100 mL) at 0 °C was added MsCl (3.1 g, 27 mmol). The resulting solution was stirred at rt for 40 min and then quenched with saturated aq. $NaHCO_3$. Usual work-up and purification on silica gel column with 5% EtOAc in hexanes yielded 14 g (90%) of the mesylate. The mesylate (41 g, 55 mmol) was dissolved in 1.0 M TBAF in THF (500 mL) and stirred at 70-80 °C for 3 days when LCMS showed that half of the starting material was converted to the desired product. Usual work-up and purification on silica gel column using 10% EtOAc in hexanes afforded 9.9 g (27%) of fluoromethyl nucleoside **10.** MS, m/z 667 $(M + 1)^+$. Obtained compound **10** (6.3 g, 9.45 mmol) and CAN (15.5 g, 28.3 mmol) was stirred overnight at rt. Usual

work-up and chromatography on silica gel using 20% EtOAc in hexanes gave N-deprotected compound (3.6 g, 71%) as a white solid. To a solution of the product from previous step (2.4 g, 4.4 mmol) in anhydrous DCM (10 mL) was added dropwise BCl_3 (1 M in DCM, 30 mL) at -70 ^{o}C. The reaction mixture was stirred at the same temperature for 2 h and then quenched with MeOH. Usual work-up and purification on silica gel using 50% EtOAc in hexanes afforded **11**[12] (1.05 g, 86%) as a white solid. MS, m/z 276.9 $(M + 1)^+$.

5'-Deoxy-5'-iodo-2'-*C*-(fluoromethyl)uridine (12). To a solution of PPh_3 (3.37 g, 12.8 mmol) in pyridine (15 mL) at 0°C was added iodine (3.06 g, 12 mmol). The mixture was stirred at rt for 30 min and then cooled to 0 ^{o}C when solution of nucleoside **11** (2.2 g, 8 mmol) in pyridine (5 mL) was added. The reaction mixture was stirred overnight at rt and then quenched with saturated aq. $Na_2S_2O_3$. Usual work-up and chromatography on silica gel using 1-2% MeOH in DCM gave **12** (1.8 g, 58%) as a white solid. MS, m/z 387.0 $(M + 1)^+$.

1-((2R,3R,4S)-3-(Fluoromethyl)-3,4-dihydroxy-5-methylenetetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (13). A solution of iodide **12** (1.35 g, 3.5 mmol) and DBU (1.06 g, 7 mmol) in THF/CH_3CN (2/1, v/v, 35 mL) was stirred at 60-70 ^{o}C for 2 h. The reaction mixture was concentrated and then dissolved in EtOAc (20 mL), washed with 10% aqueous HCl, brine and dried ($MgSO_4$). The evaporated residue was purified on silica gel using 30% EtOAc in hexanes to give olefin **13** (0.5 g, 55%) as a foam. MS, m/z 259.1 $(M + 1)^+$.

5'-Deoxy-5'-iodo-4'-fluoro-2',3'-di-*O*-benzoyluridine (16b). To a stirred solution of olefin **14**[18] (12.0 g, 53 mmol) in anhydrous CH_3CN at 0 ^{o}C was added TEA-3HF (8.5 g, 53 mmol) and NIS (10.2 g, 63.6 mmol). The reaction mixture was stirred for 30 min at 0 ^{o}C and then another 30 min at rt. Precipitate was removed by filtration and washed with DCM. The filtrate was concentrated

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to give 5'-iodo-4'-fluoro uridine analogue (14 g, 73%) as a yellow solid. MS, m/z 373.0 $(M + 1)^+$. A solution of the compound prepared in previous step (12.0 g, 32 mmol), Bz_2O (21.7 g, 96 mmol), and DMAP (1.2 g, 9.6 mmol) in pyridine (100 ml) was stirred at 50 ^oC overnight. The reaction mixture was quenched with water. Usual work-up and purification on silica gel column with 50% EtOAc in hexanes to yielded **16b** (15 g, 81%) as a white solid. MS, m/z 581.0 $(M + 1)^+$.

5'-Deoxy-5'-iodo-4'-fluoro-2',3'-di-*O*-acetyl-2'-*C*-methyluridine (16c). A solution of **15**[19] (12.0 g, 50 mmol) in pyridine (200 mL) and acetic anhydride (14.2 mL, 150 mmol) was stirred 1d at rt. Reaction was quenched with MeOH, concentrated and the residue coevaporated with toluene. Usual work-up and column chromatography with 50% EtOAc in hexanes yielded diacetate (15.0 g, 91%) as a colorless foam. To an ice-cold solution of the diacetate (15.0 g, 46.3 mmol) in anhydrous DCM (300 mL) were added AgF (29.4 g, 231 mmol) and a solution of iodine (23.5 g, 92.6 mmol) in anhydrous DCM (1.0 L). The reaction mixture was stirred at rt for 5 h, then quenched with sat. aq. $Na_2S_2O_3$ and $NaHCO_3$. Usual work-up and purification on a silica gel with 10-30% EtOAc in hexanes gave **16c** (9.5 g, 44%) as a white solid; ^1H NMR (CD_3OD) δ 7.52 (d, J = 8.0 Hz, 1H), 6.21 (s, 1H), 5.80 (d, J = 17.2 Hz, 1H), 5.73 (d, J = 8.0 Hz, 1H), 3.58 (s, 1H), 3.54 (d, J = 6.8 Hz, 1H), 2.17 (s, 3H), 2.09 (s, 3H), 1.58 (s, 3H).

4'-Fluoro-2',3'-di-*O*-benzoyluridine (17b). Tetrabutylammonium hydroxide (288 mL, 54-56% aqueous solution, 0.58 mol) was adjusted to pH 4 by adding TFA (48 ml). The resulting solution was added to a solution of **16b** (14 g, 24 mmol) in DCM (200 ml). *m*-Chloroperbenzoic acid (30 g, 60-70%, ~120 mmol) was added in portions under vigorous stirring and the resulting reaction mixture was stirred overnight. Usual work-up and chromatography on silica gel with 4% MeOH in DCM gave **17b (**7.5 g, 54%) as off-white solid.

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4'-Fluoro-2'-*O*-acetyl-3'-5-di-*O*-benzoyl-2'-*C*-methyluridine (17c). A mixture of **16c** (7.0 g, 14.9 mmol), NaOBz (21.44 g, 149 mmol) and 15-crown-5 (32.75 g, 149 mmol) in anhydrous DMF (400 mL) was stirred at 130 ^oC for 6 h and then concentrated. Usual work-up and purification on a silica gel with 10-30% EtOAc in hexanes gave **17c** (2.8 g, 41%). ^1H NMR ($CDCl_3$) δ 8.84 (s, 1H), 8.04-8.06 (m, 2H), 7.59 (t, J = 7.2 Hz, 1H), 7.44-7.47 (m, 2H), 7.21-7.26 (m, 1H), 6.21 (s, 1H), 5.85 (d, J = 18 Hz, 1H), 5.67 (d, J = 8.0 Hz, 1H), 4.59-4.72 (m, 2H), 2.14 (s, 6H), 1.64 (d, J = 6.0 Hz, 3H). MS, m/z 527.2 $(M + 1)^+$.

4'-Fluorouridine (18). A solution of **17b** (5.0 g, 8.7 mmol) in methanolic ammonia (7 M, 100 mL) was stirred at rt for 5 h and then concentrated. The solid crude product was filtered and washed with DCM to give **18** (2.1 g, 92%) as white foam. ^1H-NMR (CD_3OD) δ= 7.71 (d, J = 7.6 Hz, 1H), 6.05 (s, 1H), 5.68 (d, J = 7.6 Hz, 1H), 4.39-4.45(m, 1H), 4.25 (d, J = 7.6 Hz, 1H), 3.72-3.73(m, 2H). MS, m/z 263.0 $(M + 1)^+$.

2',3'-di-*O*-Benzoyl-2'-*C*-ethynyl-4'-fluoro-N^3-benzoyluridine (25). A solution of TsOH·H_2O (0.7 g, 3.7 mmol), 1,1-dimethoxycyclopentane (19.3 g, 148.5 mmol) and 2'-ethynyluridine **1c**[14] (10.0 g, 37.1 mmol) in DCE (100 mL) was stirred at 50 ^oC overnight. The reaction mixture was neutralized with Et_3N and concentrated. The residue was purified on silica gel (1-10% MeOH in DCM) to give **21** (8.7 g, 69%) as a white solid. MS, m/e 335.0 $(M+1)^+$. To an ice-cold solution of **21** (20.0 g, 0.06 mol) in anhydrous pyridine (100 mL) was added TsCl (22.8 g, 0.12 mol). The reaction mixture was stirred overnight, then quenched with water. A usual work-up and purification on silica gel (1-6% MeOH in DCM) produced 5'-*O*-tosyl nucleoside (20.0 g, 69%) as a white solid. A solution of tosylate (20.0 g, 0.04 mol) and NaI (31.0 g, 0.2 mol) in acetone (200 mL) was refluxed overnight and then quenched with saturated $Na_2S_2O_3$ solution. Usual work-up and chromatography on silica gel (1-6% MeOH in DCM) gave iodide **22** (15.0 g, 56%) as a white

solid. MS, m/e 443.1 $(M-1)^-$. Compound **22** (13.4 g, 30.2 mmol) in 50 ml of 80% aq. AcOH was stirred at 60 °C for 2 h. The reaction mixture was concentrated and the residue purified on silica gel (1-10% MeOH in DCM). The product (5.0 g, 13.22 mmol) was treated as described for **13** to give the olefin **23** as a white solid. After iodofluorination of **23,** as described for **16b**, with subsequent benzoylation the product **24** (1.8 g, 19 %) was obtained as a white solid. MS, m/z 709.1 $(M+1)^+$. Compound **24** (600 mg, 0.85 mmol) in DCM (10 mL) yielded **25** (123 mg, 24%) as a white solid as described for **17b**. MS, m/z 599.1 $(M + 1)^+$.

(4S,5R)-ethyl-5-((R)-2,2-dimethyl-1,3-dioxolan-4-yl)-4-methyl-1,3,2-dioxathiolane-4-carboxylate 2,2-dioxide (29). TBACl (102.5g, 370 mmol) was added to a solution of **28**[21] (30.0 g, 95 mmol) in anhydrous dioxane (1 L), and the mixture was heated at 120 °C overnight. After cooling to rt. 2,2-dimethoxypropane (300 mL) and conc. hydrochloric acid (20 mL) were added. The mixture was stirred for 3 h at rt, then evaporated to 1/3 of the volume, diluted with EtOAc and then washed with cold saturated sodium bicarbonate and brine. The combined aqueous layer was extracted with EtOAc (2 x 100 mL). The combined organic extract was dried (Na_2SO_4), filtered, and concentrated to give **29** (22 g, 71%). ^{1}H-NMR (DMSO-d_6), δ 4.18-4.03 (m, 2H), 3.90-3.82(m, 1H), 3.15-3.32 (m, 2H), 1.50-1.57 (m, 2H), 1.18-1.40 (m, 9H), 0.92-0.96 (m, 3H).

1-*O*-Acetyl-2-deoxy-2-chloro-3,5-di-*O*-benzoyl-2-*C*-methyl-α/β-D-ribofuranose (31). The ester **29** (22 g, 82.7 mmol) was dissolved in ethanol (200 mL) and conc. hydrochloric acid (6 mL). The resulting solution was stirred at rt for 48 h, then concentrated under reduced pressure, the residue co-evaporated with toluene to give the unprotected lactone as a pale yellow solid (10.7 g). The unprotected lactone was dissolved in anhydrous pyridine (100 mL) and benzoyl chloride (20 mL, 167 mmol) was added dropwise at 0-5 °C. The resulting mixture was stirred at rt for 45 min, then quenched with ice and MeOH to form a suspension. The solid was filtered and washed with

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MeOH to give ribonolacton **30** (18.5 g, 56.6%) as white solid. The lactone **30** (2.3 g, 5.9 mmol) was dissolved in anhydrous THF (50 mL) and the solution was cooled to -60 °C under nitrogen. Lithium tri(*tert*-butyl)aluminum hydride (1.0 M in THF, 20 mL) was added over 3 min with stirring at -60 °C. After 3 h at the same temperature, the mixture was quenched and diluted with saturated aqueous NH_4Cl (30 mL). Resulting precipitate was filtered and washed with EtOAc (3 x 50 mL). The combined organic extract was concentrated and re-dissolved in THF (50 mL). DMAP (80 mg, 0.66 mmol) and acetic anhydride (5.46 g, 53.5 mmol) were added at 0°C. The mixture was stirred at 0-5 °C for 1 h, concentrated, and the residue was purified on silica gel with 35% EtOAc in hexanes to give acetate **31** (2.0 g, 79%) as a white solid. ^{1}H-NMR ($CDCl_3$), δ 7.54-8.11 (m, 10H), 6.35 (s, 1H), 5.78 (d, *J* = 8.0 Hz, 1H), 4.65-4.74 (m, 2H), 4.43 (dd, *J* = 11.6 Hz, 4.4Hz, 1H), 1.97 (s, 3H), 1.69 (s, 3H).

2'-Deoxy-2'-chloro-2'-*C*-methyl-3',5'-di-*O*-benzoyl-*N*^{4}-benzoylcytidine (32). A suspension of N^4-benzoylcytosine (2.3 g, 11 mmol) and ammonium sulfate (45 mg) in hexamethyldisilazane (30 mL) was refluxed for 4 h and the resulting solution was concentrated, followed by drying under vacuum for 3 h. The resulting silylated cytosine was dissolved in chlorobenzene (30 mL). To this stirred solution was added riboside **31** (2.0 g, 4.6 mmol) and tin(IV) chloride (2.4 mL, 10.5 mmol). After stirring at rt for 2 h, the reaction mixture was heated at 60-70 °C overnight, then cooled to 0 °C, and quenched with solid sodium bicarbonate (4 g) , followed by slow addition of water (20 mL). In 20 min precipitate was filtered and washed with EtOAc. Usual work-up and purification by column chromatography (5-35% EtOAc in hexanes) afforded nucleoside **32** (1.2 g, 44.6%) as a white solid. ^{1}H-NMR ($CDCl_3$), δ 8.82 (br, s, 1H), 7.56-8.22 (m, 17H), 6.72 (s, 1H), 5.59 (d, *J* = 8.8 Hz, 1H), 4.86-4.90 (m, 1H), 4.78-4.82 (m, 1H), 4.64 (dd, J_1= 13.2 Hz, J_2 =3.2 Hz, 1H), 1.61 (s, 3H). MS, m/e 588 $(M+1)^+$.

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3,5-bis-O-(triisopropylsilyl)-2-deoxy-2-fluoro-2-chloro-α and β-D-ribofuranosylbromide (35a and 35b). To a solution of **34**[23] (4.00 g, 8.0 mmol) and PPh_3 (6.30 g, 24.0 mmol) in DCM (32.0 mL) was added CBr_4 (8.77 g, 26.4 mmol) at 0 °C. The resulting solution was stirred at 25 °C for 1 h and then concentrated. The residue was purified on silica gel with 1-3% EtOAc in hexanes to afford stereoisomers **35a (**3.6 g, 45% yield) and **35b** (2.11 g, 47% yield) as yellow oils. **35a**: ^{1}H-NMR ($CDCl_3$), δ 6.54 (s, 1H), 4.70 (d, *J* = 4.6 Hz, 1H), 4.03 (d, *J* = 4.6 Hz, 1H), 3.98 (dd, *J* = 4.6 Hz, 8.1 Hz, 2H), 1.09 (m, 42H). **35b**: ^{1}H-NMR ($CDCl_3$), δ 6.44 (d, *J* = 4.6 Hz, 1H),5.01 (dd, *J* = 8.6 Hz, 16.1 Hz, 1H), 4.11 (t, *J* = 4.6 Hz, 1H), 3.95 (dd, *J* = 4.6, 8.1Hz, 2H), 1.11 (m, 42H).

4'-Fluoro-2'-*C*-methyluridine (2a). A mixture of **17c** (4.0 g, 8.6 mmol) and liquid ammonia in a stainless steel vessel was kept overnight at rt. Ammonia was let evaporate and the residue purified on silica gel with 4-12% MeOH in DCM to yield target **2a** as a colorless foam (2.0 g, 84%). ^{1}H NMR (CD_3OD) δ 7.89 (d, *J* = 8.1 Hz, 1H), 6.27 (s, 1H), 5.70 (d, *J* = 8.1 Hz, 1H), 4.02 (d, *J* = 19.8 Hz, 1H), 3.76-3.82 (m, 2H), 1.19 (s, 3H). ^{13}C-NMR (DMSO-d_6) δ 163.23, 150.93, 139.96, 117.24 (d, *J* = 231.9 Hz), 102.54, 92.94, 76.60, 72.83 (d, *J* = 19.7 Hz), 59.48 (d, *J* = 45.0 Hz), 21.34. ^{19}F NMR (CD_3OD) δ -124.22 (m). MS, m/z 275.1 $(M – 1)^-$

4'-Fluoro-2'-*C*-fluoromethyluridine (2b). Olefin **13** (670 mg, 2.6 mmol) yielded 1.2 g (80%) of the iodofluo nucleoside as described for **16b**. Subsequent benzoylation (1.0 g, 2.47 mmol) gave 850 mg (~80% purity, 45%) of **16a**. MS, m/z 612.9 $(M + 1)^+$. A solution of **16a** (600 mg, ~80% purity, 0.78 mmol), NaOBz (1.45 g, 10 mmol) and 15-crown-5 (2.2 g, 10 mmol) in DMF (25 mL) was stirred at 90-100 °C for 24 h. After evaporation and usual work-up the residue was purified on silica gel with 15% EtOAc in hexanes to give benzoylated nucleoside **17a** (275 mg, 58%) as a light-yellow foam. MS, m/z 607.1 $[M + 1]^+$. Treatment of **17a** (250 mg, 0.41 mmol) with

methanolic ammonia gave, after purification by RP-HPLC, **2b** (33 mg, 27%) as a white solid. ^{1}H-NMR (CD_3OD), δ 7.90 (d, J = 8.0 Hz, 1H), 6.37 (s, 1H), 5.69 (d, J = 8.4 Hz, 1H), 4.51-4.28 (m, 3H), 3.82 (m, 2H), 3.68 (s, 0.5H). ^{19}F-NMR (CD_3OD), δ = -123.68 (s, 1F), -227.41 (s, 1F). MS, m/z 295.1 $(M + 1)^+$.

2'-*C*-Ethynyl-4'-fluorouridine (2c). To a solution of 4'-fluorouridine **18** (2.1 g, 8.0 mmol) in pyridine at 0 ºC was added dropwise $TIPDSCl_2$ (2.5 g, 8.0 mmol). The resulting solution was stirred at rt overnight, then quenched with water. Usual work-up and purification on silica gel with 10% to 50% EtOAc in hexanes gave **19** (1.6 g, 40%) as a white foam. A solution of **19** (1.5 g, 3.0 mmol) and IBX (1.69 g, 6.0 mmol) in anhydrous CH_3CN (10 mL) was stirred at 80 ºC for 3 h. The reaction mixture was cooled down to rt and precipitate filtered. The filtrate was concentrated to dryness. Chromatography on silica gel using 20% to 50% EtOAc in hexanes afforded ketone **20** (1.2 g, 80%) as a white foam. ^{1}H NMR ($CDCl_3$) δ 6.23 (s, 1H), 7.16 (d, J = 8.0 Hz, 1H), 5.81 (s, 1H), 5.46 (d, J = 8.0 Hz, 1H), 5.17 (s, 1H), 4.02-4.08 (m, 2H), 1.07-1.13 (m, 28H). MS, m/z 503.0 $(M + 1).^+$ To a solution of **20** (500 mg, 1 mmol) in 8 mL of anhydrous THF under nitrogen was added ethynylmagnesium bromide (0.5 M in cyclohexane, 8 mL) at rt. After stirring for 30 min, another portion (8 mL) of the Grignard reagent was added. Stirring was continued for 30 min more and then reaction quenched with sat. aq. NH_4Cl. After usual work-up the residue was purified on silica gel with EtOAc. A solution of the resulting product in THF (3 mL) and TBAF (1 mL, 2 M in THF) was kept at rt for 30 min. Solvent was evaporated and the residue was purified on silica gel with EtOAc saturated with water to give 110 mg (38%) of **2c** as off-white solid. ^{1}H-NMR (DMSO-d_6) δ 11.44 (s, 1H), 7.64 (d, J = 8.2 Hz, 1H), 6.32 (s, 1H), 6.15 (s, 1H), 5.98 (br, 1H), 5.76 (br, 1H), 5.67 (d, J = 8.2 Hz, 1H), 4.23 (d, J = 20.0 Hz, 1H), 3.57 (m, 2H), 3.46 (s, 1H). ^{13}C-NMR (DMSO-d_6) δ 59.38 (d, J = 43.7 Hz), 74.18, 74.68 (d, J = 19.8 Hz), 78.09, 82.0, 92.36,

102.44, 117.06 (d, J = 233.5 Hz), 139.92, 150.64, 163.27. ^{19}F-NMR (DMSO-d$_6$) δ -120.34 (m); MS: 285.1 (M-1)$^-$.

2'-*C*-Ethenyl-4'-fluorouridine (2d). A mixture of compound **25** (300 mg, 0.50 mmol) and Lindlar catalyst (200 mg) in 20 mL of EtOAc/hexanes (1:1, v:v) was stirred under hydrogen (40 psi) at rt for 1.5 h. The catalyst was removed by filtration, and a mixture of the filtrate and fresh Lindlar catalyst (200 mg) was stirred under hydrogen (40 Psi) at 25 ^{o}C for another 1.5 h. Solid was filtered, and the filtrate was concentrated to give the crude **26.** Deprotection with methanolic ammonia resulted in **2d** (50 mg, 35% over two steps) as a white solid. ^{1}H-NMR (CD$_3$OD) δ 7.86 (d, J = 8.0 Hz 1H), 6.26 (s, 1H), 5.86-5.62 (m, 2H), 5.49 (d, J = 17.1 Hz, 1 H), 5.30 (d, J = 10.5 Hz, 1H), 4.41 (d, 1 H J = 19.3 Hz,), 3.71-3.86 (m, 1H). MS, m/e 289.1 (M+1)$^+$.

2'-C-Ethyl-4'-fluorouridine (2e). A mixture of **25** (300 mg, 0.50 mmol) and Pd/C (300 mg, 10%) in 30 mL of MeOH was stirred under hydrogen (1 atm) at 25 ^{o}C for 1.5 h. The suspension was filtered and the filtrate concentrated to give the crude 2'-C-ethyl nucleoside **27**. Subsequent deprotection by methanolic ammonia gave **2e** (30 mg, 21% over two steps) as a white solid. ^{1}H-NMR (CD$_3$OD) δ 7.77 (d, J = 8.0 Hz, 1H,), 6.32 (s, 1H), 5.71 (d, J = 8.0 Hz, 1H), 4.14 (d, J = 16.0 Hz, 1H,, 3.75-3.73 (m, 2H), 1.54-1.49 (m, 2H), 0.98 (t, 3H). MS, m/e 291.1 (M+1)$^+$.

2'-Deoxy-2',4'-difluoro-2'-*C*-methyluridine (3b). As described for **12**, a solution of **3a**[26] (260 mg, 1 mmol), PPh$_3$ (780 mg, 3 mmol), iodine (504 mg, 2 mmol) and pyridine (0.5 mL) in anhydrous THF (8 mL) was stirred at rt overnight. Usual work-up and purification on silica gel with 5% MeOH in DCM yielded **40a** (190 mg, 51%) as a white solid. MS, m/z 371.1 (M+1)$^+$. as described for the preparation of **13**, a solution of **40a** (190 mg, 0.52 mmol) and DBU (760 mg, 5 mmol) in THF (4 mL) was heated at 50 ^{o}C overnight. Usual work-up and purification on silica gel

with 30% EtOAc in hexanes gave the product **41a** (75 mg, 60%) as white solid. MS, m/z 242.2 $(M+1)^+$. A solution of **41a** (200 mg, 0.82 mmol), NIS (337 mg, 1.5 mmol), and TEA-3HF (213 mg, 1.3 mmol) in MeCN (4 mL) was stirred at rt for 7 h. Usual work-up and purification on silica gel with 20% EtOAc in hexanes afforded 5'-iodo-4'-F uridine analogue (300 mg, 94%) as a white solid. A solution of the iodo-fluoro product from previous step (194 mg, 0.5 mmol) and BzCl (92 mg, 0.55 mmol) in pyridine (5 mL) was stirred at rt for 5 h. Usual work-up and purification on silica gel with 20% EtOAc in hexanes gave **42a** (199 mg, 81%) as white solid. MS, m/z 493.4 $(M+1)^+$. A solution of **42a** (1.05 g, 2.13 mmol) and a mixture of TFA (0.5 mL) and Bu_4NOH (1 mL) in DCM (12 mL) was stirred at rt for 5 h. Usual work-up and purification on silica gel with 30% EtOAc in hexanes gave **43a** (450 mg, 55%) as a white solid. MS, m/z 383.2 $(M+1)^+$. A solution of **43a** (250 mg, 0.65 mmol) in 7 M ammonia in MeOH (5 mL) was kept at rt for 5 h. After removal of volatiles, the residue was purified on silica gel with 5% MeOH in DCM to yield **3b** (120 mg, 66%) as a white powder. ^{1}H-NMR (CD_3OD) δ 7.85 (d, J = 7.0 Hz, 1H), 6.40-6.55 (m, 1H), 5.75 (d, J = 7.0 Hz, 1H), 4.05-4.25 (m, 1H), 3.81 (s, 2H), 1.41 (d, J = 22 Hz, 3H). ^{13}C-NMR (DMSO-d_6) δ 163.14, 150.89, 139.05, 117.05 (d, J = 232.7 Hz), 103.17, 98.60 (d, J = 184.6 Hz), 89.70 (d, J = 42.7 Hz), 71.73 (m), 58.99 (d, J = 44.3 Hz), 17.61 (d, J = 25.9 Hz). MS, m/z 279.0 $(M + H)^+$.

2'-Deoxy-2',4'-difluoro-2'-*C*-ethynyluridine (3d). Similar for the preparation of **12**, a stirred suspension of 2'-ethynyl-2'-fluorouridine **3c**[25] (4.1 g, 15.2 mmol), PPh_3 (8.0 g, 30.4 mmol), iodine (5.8 g, 22.8 mmol), imidazole (2.1 g, 30.4 mmol) and pyridine (18.2 mL) in THF (40 mL) was stirred at rt overnight. Usual work-up and purification on silica gel with 4% MeOH in DCM) yielded iodide **40b** (4.4 g, 77%) as a white solid. ^{1}H NMR (CD_3OD) δ 7.73 (d, J = 8.4 Hz, 1H), 7.73 (d, J = 19.2 Hz, 1H), 5.75 (d, J = 8.4 Hz, 1H), 4.00-4.06 (dd, J = 8.0 Hz, 16.0 Hz, 1H), 3.67-

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3.74 (m, 2H), 3.58 (d, 8 Hz, 1H), 3.50-3.54 (m, 1H); MS: m/z 381.1 $(M + 1)^+$. As described for **13** solution of **40b** (2.5 g, 6.6 mmol) and DBU (2.1 g, 14 mmol) in THF (3 mL) was stirred at rt for 1 h. Usual work-up and purification on silica gel with 5% MeOH in DCM afforded olefin **41b** (1.1 g, 69%) as a white foam. MS, m/z 253.3 $(M + 1)^+$. A solution of **41b (**800 mg, 3.17 mmol), TEA-3HF (510 mg, 3.17 mmol), and NIS (785 mg, 3.49 mmol) in CH_3CN (10 mL) was stirred at 0 °C for 30 min and at rt for 1 h. Usual work-up and purification on silica gel gave the 5'-iodo-4'-F uridine analogue (695 mg, 55%) as a yellow solid. A solution of the 5'-iodo-4'-F intermediate (650 mg, 1.63 mmol) and BzCl (507 mg, 3.59 mmol) in pyridine (3 ml) was stirred overnight at rt. Usual work-up and purification on silica gel with 50% EtOAc in hexanes afforded benzoate **42b** (550 mg, 67%) as a white foam. MS, m/z 503.0 $(M + 1)^+$. Compound **42b** (375 mg, 0.75 mmol) yielded **43b** (230 mg, 78%) as described for **17b**. ^{1}H NMR ($CDCl_3$) δ 9.08 (s, 1H), 8.17 (d, *J* = 1.6 Hz, 2H), 7.49-7.69 (m, 4H), 6.63 (s, 1H), 5.30-5.91 (m, 3H), 4.11 (d, *J* = 6.8 Hz, 1H), 4.00 (d, *J* = 6.8 Hz, 1H) 2.92 (d, *J* = 5.2 Hz, 1H). MS, m/z 393.1 $(M + 1)^+$. After deprotection by ammonolysis **43b** (120 mg, 0.31 mmol) yielded **3d** (53 mg, 59%) as a white solid. ^{1}H NMR (CD_3OD) δ = 7.73 (d, *J* = 8.4 Hz, 1H), 6.51 (s, 1H), 5.74 (d, *J* = 8.4 Hz, 1H), 4.51(m, 1H), 3.78 (d, *J* = 3.2 Hz, 2H), 3.57 (d, 5.6 Hz, 1H). ^{13}C-NMR (DMSO-d_6) δ 163.12, 150.49, 138.64, 116.96 (d, *J* = 235.0 Hz), 103.21, 93.05 (d, *J* = 188.5 Hz), 89.32 (d, *J* = 40.4 Hz), 83.56, 76.12 (d, *J* = 31.3 Hz), 74.02 (m), 58.88 (d, *J* = 38.9 Hz). ^{19}F-NMR (DMSO-d_6) δ -123.50, -154.97. MS: m/z 288.8 $(M + 1)^+$.

2'-Chloro-2'-deoxy-2'-*C*-methyluridine (4a). A solution of protected nucleoside **32** (15.0 g, 25.6 mmol) in a mixture of acetic acid/water (90:10, 150 mL) was heated at 110 °C overnight, and then concentrated. After usual work-up, the residue was purified on silica gel (5% MeOH in DCM) to give uridine **33** (11.0 g, 89%) as a white solid. Nucleoside **33** (1.2 g, 2.0 mmol) was dissolved in

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methanolic ammonia NH_3 (10 M), kept at rt overnight and evaporated. The residue was purified on silica gel (2-10% MeOH in DCM) to give **4a** (0.43g, 76.6%) as white solid. ^{1}H-NMR (DMSO-d_6), δ 8.14 (d, J = 8.2 Hz, 1H), 6.19 (s, 1H), 5.94 (br, 1H), 5.90 (d, J = 8.2 Hz, 1H), 5.40 (br, 1H), 3.80-3.88 (m, 3H), 3.61-3.64 (m, 1H), 1.40 (s, 3H). ^{13}C-NMR (DMSO-d_6), δ 163.37, 151.16, 139.91, 102.18, 91.61, 82.80, 78.97, 71.92, 58.36, 22.72. MS, m/e 277.0 $(M+1)^+$.

2'-Chloro-2'-deoxy-4'-fluoro-2'-*C*-methyluridine (4b). 2'-Methyl-2'chloro uridine **4a**[20] (4.3 g, 15.9 mmol) yielded iodide **40c** (5.1 g, 85%) as a white solid as described for **12**. MS: m/z 387.3 $(M + 1)^+$. **40c** (800 mg, 2.1 mmol) was converted to olefin **41c** (240 mg, 45%) as described for **13**. MS, m/z 259.4 $(M + 1)^+$. **41c** (1.20 g, 4.6 mmol) was treated as described for **16b** to yield the 5'-iodo-4'-F **42c** (0.91 g, 48%). This intermediate (1.09 g, 2.14 mmol) yielded **43c** (350 mg, 41%) as described for **17b**. MS, m/z 399.3 $(M + 1)^+$. Ammonolysis of **43c** (280 mg, 0.70 mmol) yielded **4b** (110 mg, 53%) as a white solid. ^{1}H-NMR (CD_3OD) δ 7.99 (d, J = 8.0 Hz, 1H), 6.65 (s, 1H), 5.72 (d, J = 8.0 Hz, 1H), 4.26 (d, J = 18.8 Hz, 1H), 3.72-3.83 (m, 2H), 1.54 (s, 3H). ^{13}C-NMR (DMSO-d_6) δ 163.12, 150.93, 139.07, 117.18 (d, J = 234.3 Hz), 102.91, 92.88, 73.45, 17.17 (d, J = 19.8 Hz), 59.08 (d, J = 45.8 Hz), 24.43. ^{19}F NMR (CD_3OD) δ = -123.96. MS, m/z 295.1 $(M + 1)^+$.

2'-Chloro-2'-deoxy-2'-fluorouridine (5c). A solution of a α/β-mixture of bromides **35a** and **35b** (5.10 g, 9.1 mmol) and NaN_3 (12.46 g, 191.7 mmol) in DMF (40 mL) was stirred at 50 °C overnight. The reaction mixture was diluted with H_2O (30 mL) and extracted with EtOAc. Usual work-up and purification on silica gel with 1-2.5% EtOAc in hexanes afforded α/β-mixture of azides **36** (4.21 g, 89% yield) as a yellow oil. A suspension of azides **36** (5.90 g, 11.3 mmol) and 20% $Pd(OH)_2$/C (1.5 g) in EtOAc (50 mL) was stirred under hydrogen (15 psi) at rt for 2 h. The catalyst was filtered and the filtrate was concentrated. The residue was purified on silica gel with 1-10% EtOAc in hexanes to afford α/β-mixture of amines **37** (5.50 g, 98%) as a yellow oil. MS, m/z 498.2

$(M+1)^+$. To a solution of amines **37** (8.1 g, 16.3 mmol) in DCM (100 mL) at -40 °C was added (*E*)-3-ethoxyacryloyl isocyanate (6.8 g, 48.3 mmol) in toluene (120 mL). The reaction mixture was stirred at rt for 2 h and then concentrated. The residue was purified on silica gel with 3-17% EtOAc in hexanes to afford α/β-mixture **38** (9.80 g, 94%) as a colorless oil. MS, m/z 639.1 $(M + 1)^+$**.** To a solution of **38** (9.80 g, 15.3 mmol) in EtOH (200 mL) was added aq. HCl (2.0 M, 50 mL). The resulting mixture was heated at 85 °C for 10 h then cooled to rt and neutralized with aq. ammonia. The mixture was concentrated and dissolved in MeOH (20 mL). NH_4F (8.52 g, 229.9 mmol) was added and the resulting solution was heated at 70 °C for 5 h. After removal of solvent, the residue was purified on silica gel with 1-5% MeOH in DCM to give α/β-mixture of uridine analogue **5a**[22] (3.20 g, 74%) as a white solid. MS, m/z 281.0 $(M+1)^+$. Pure β-isomer **5c** was obtained by HPLC separation of **5a** and was identical to the published data.[23]

2'-Chloro-2'-deoxy-2',4'-difluorouridine (5b). 5a[22] (α/β-mixture, 7.20 g, 25.7 mmol) yielded α/β-mixture of **40d** as described for **12.** Purification by RP-HPLC (column: Agela Durashell C18 150 x2 5mm, 5μ; mobile phase: A, 0.2% aq. HCOOH, B 0.2% HCOOH in MeCN; gradient 5%-35% B in 12.2 min) afforded **40d-**β isomer (2.7 g, 27%) as a white solid. ^{1}H-NMR (CD_3OD), δ 7.69 (d, *J* = 8.4 Hz, 1H), 6.33 (d, *J* = 8.0 Hz, 1H), 5.77 (d, *J* = 8.4 Hz, 1H), 4.15-4.03 (m, 1H), 3.75-3.61 (m, 1H), 3.7-3.45 (m, 2H). MS, m/z 390.7 $(M + 1)^+$. As described for **13**, **40d-**β isomer (2.10 g, 5.4 mmol) yielded **41d** (1.10 g, 78% yield) as a yellow solid. ^{1}H NMR (CD_3OD) δ 7.51 (d, *J* = 8.2 Hz, 1H), 6.45 (br d, *J* = 12.3 Hz, 1H), 5.75 (d, *J* = 8.2 Hz, 1H), 5.04 - 4.90 (m, 1H), 4.74 - 4.67 (m, 1H), 4.51 (s, 1H). B. **41d** (1.10 g, 4.2 mmol) yielded the 5'-iodo-4'-F intermediate (1.10 g, 64%) as a yellow solid as described for **16b**. ^{1}H-NMR (CD_3OD), δ = 7.60 (d, *J* = 8.4 Hz, 1H), 6.54-6.48 (m, 1H), 5.80 (d, *J* = 8.4 Hz, 1H), 4.77-4.68 (m, 1H), 3.71-3.69 (m, 2 H). MS, m/z 408.7 $(M + 1)^+$. Benzoylation of the fluoro-iodo intermediate from previous step (1.10 g, 2.7 mmol)

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yielded **42d** (855 mg, 62%) as colorless oil. ^{1}H-NMR ($CDCl_3$), δ 8.81 (s, 1H), 8.15-8.17 (m, 2H), 7.69-7.65 (m, 1H), 7.64-7.50 (m, 2H), 7.48-7.40 (m, 1H), 6.48-6.21 (m, 2H), 5.91-5.88 (m, 1H), 3.72-3.58 (m, 2 H). MS, m/z 512.8 $(M + 1)^+$. **42d** (400 mg, 0.78 mmol) was converted to **43d** (179 mg, 57% yield) as described for **17b**. MS, m/z 403.0 $(M + 1)^+$. Ammonolysis of **43d** (150 mg, 0.37 mmol) yielded **5b** (89 mg, 81%) as a white solid. ^{1}H-NMR (CD_3OD), δ 7.70 (d, J = 8.4 Hz, 1H), 6.58 (d, J = 14.4 Hz, 1H), 5.76 (d, J = 8.4 Hz, 1H), 4.58-4.49 (m, 1H), 3.78 (d, J = 3.6 Hz, 2H). ^{13}C-NMR (DMSO-d_6) δ 163.24, 150.53, 138.58, 116.0 (d, J = 236.5 Hz), 112.75 (d, J = 254.82 Hz), 103.51, 88.52, 74.75 (m), 59.09 (d, J = 35.1 Hz). ^{19}F-NMR (DMSO-d_6) δ -115.42 (br), -124.99 (br). MS, m/z 298.9 $(M + 1)^+$.

General method for preparation of phosphoramidates[29]

Dry nucleosides (1.0 mmol) was dissolved in the mixture of dry acetonitrile 4.5 mL) and N-methylimidazole (0.5 mL). The appropriated phosphorochloridate was added (4 equiv.) and the reaction mixture was kept overnight at rt or heated up to 70 °C for 2-10h; disappearance of starting nucleoside was controlled by LCMS. After the reaction was completed it was diluted with EtOAc (30 mL). Organic phase was washed with 10% citric acid, brine, dried (Na_2SO_4) and reduced to dryness. The residue was purified on silica gel with gradient of MeOH in DCM from 2% to 10%. Prodrugs were obtained as mixtures of Rp and Sp isomers with total yield 50-85%.

Separation of Rp and Sp-isomers of compound 44

Compound **44** was separated by RP HPLC on Synergy 4-micron Hydro-RP 80A (250 x 30 mm) column (Phenominex). A linear gradient of CH_3CN in water from 10% to 43% in 26 min with flow

rate 24 ml/min was used for elution. Two compounds were eluted. The corresponding fractions were combined and concentrated.

Compound **53** with retention time 30.2 min was assigned as Sp-isomer, it structure was determined by X-ray crystal structure. ^{1}H NMR (DMSO-d_6) δ 11.51 (s, 1H), 7.48-7.35 (m, 3H), 7.25-7.18 (m, 3H), 6.17-6.08 (m, 2H), 5.57-5.50 (m, 2H), 5.34 (s, 1H), 4.90-4.80 (m, 1H), 4.23-4.20 (m, 2H), 4.05-3.93 (m, 1H), 3.87-3.78 (m, 1H), 1.23 (d, *J* = 7.2 Hz, 3H), 1.15 (d, *J* = 6.0 Hz, 6H), 1.06 (s, 3H). ^{31}P NMR (DMSO-d_6) δ 3.54. MS, m/z 544.0 $(M-1)^{-}$.

Compound **54** with retention time 29.6 min was assigned as Rp-isomer. ^{1}H NMR (DMSO-d_6) δ 11.45 (s, 1H), 7.36-7.32 (m, 3H), 7.17-7.15 (m, 3H), 6.11-6.08 (m, 2H), 5.62-5.55 (m, 1H), 5.51 (d, *J* = 8.0 Hz, 1H), 5.29 (s, 1H), 4.86-4.78 (m, 1H), 4.30-4.15 (m, 2H), 3.92-3.80 (m, 1H), 3.77-3.70 (m, 1H), 1.17 (d, *J* = 6.4 Hz, 3H), 1.13-1.10 (m, 6H), 0.99 (s, 3H).). ^{31}P NMR (DMSO-d_6) δ 3.48. MS, m/z 544.0 $(M-1)^{-}$.

General method for preparation of phosphorbisamidates[31]

To the solution of appropriate ester of *S*-alanine hydrochloride (2 equiv.) and $POCl_3$ (1 equiv.) in DCM was added TEA (4 equiv.) dropwise at 0 °C and the mixture was stirred for 2h. A solution of pentafluorophenol (1 equiv.) and TEA (1 equiv.) in DCM was added dropwise at 0 °C and the mixture was stirred for 15h. After completion of the reaction the solvent was reduced, the residue was suspended in TBME, and filtered. Filtrate was concentrated and purified on silica with 20% of EtOAc in hexanes.

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The obtained phosphorochloridate (1 equiv.) was added to solution of the properly 2',3'-protected nucleoside (1.0 mmol) and tert-BuMgCl (1.0 M solution in THF, 1.2 equiv.) in dry THF. The reaction mixture was stirred for 4 h, quenched with water, and extracted with EtOAc. Organic extract was dried (Na_2SO_4), reduced to dryness and purified on silica gel with gradient of 2-5% MeOH in DCM. After deprotection of 2',3'-hydroxyls by appropriate methods the final prodrugs were purified by RP HPLC 0% to 75% B; A: 0.05% aq. HCOOH, B: 0.05% HCOOH in MeCN.

Table 8. MS and ^{31}P NMR (CD_3OD) of nucleoside prodrugs

Prodrug	P-31	MS(M-1)	Purity[a]
44	3.52; 3.47	544.1	97
45	2.54; 2.46	593.9	99
46	3.59; 3.45	556.3	99
47	3.57; 3.45	557.9	99
48	3.85; 3.74	528.3	98
49	3.51; 3.36	546.1	99
50	3.88; 3.76	545.9	99
51	2.62; 2.39	562.1	97
52	3.32, 3.57	566.3	99
53	3.54	544.0	99
54	3.48	544.0	99
55	3.49; 3.53	584.2	98
56	2.57; 2.43	572.6	99
57	3.12; 341	594.3	98
58	3.67; 3.58	579.5	99
59	12.29	553.1	95

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60	13.83	581.3	99

[a] Analytical HPLC was performed on Synergy 4-micron Hydro RP 80 A 150 x 6 mm, flow 1.5 ml/min. A linear gradient of methanol from 25% to 95 % in 50 mM triethylammonium acetate buffer (pH 7.5) was used for elution.

General method for preparation of nucleoside 5'-triphosphates

Dry nucleoside (0.05 mmol) was dissolved in $PO(OMe)_3$ (0.7 mL) N-Methylimidazole (0.009 mL, 0.11 mmol) was added followed by $POCl_3$ (0.009 mL, 0.11 mmol), and the mixture was kept at rt for 20-40 mins. The reaction was controlled by LCMS and monitored by the appearance of corresponding nucleoside 5'-monophosphate. After completion of the reaction, tetrabutylammonium salt of pyrophosphate (150 mg) was added, followed by DMF (0.5 mL) to get a homogeneous solution. After 1.5 h at rt, the reaction was diluted with water (10 mL) and loaded on the column HiLoad 16/10 with Q Sepharose High Performance. Separation was done in a linear gradient of NaCl from 0 to 1N in 50mM TRIS-buffer (pH7.5). Triphosphate was eluted at 75-80%B. Corresponding fractions were concentrated. Desalting was achieved by RP HPLC on Synergy 4-micron Hydro-RP column (Phenominex). A linear gradient of acetonitrile from 0 to 30% in 50mM triethylammonium acetate buffer (pH 7.5) was used for elution. The corresponding fractions were combined, concentrated and lyophilized 3 times to remove excess of buffer.

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Table 9. MS and ^{31}P NMR (D_2O) of nucleoside 5'-triphosphates

NTP	^{31}P NMR	MS $(M-1)^-$	Purity %[a]
1a-TP[30]	-8.30(d), -11.20(d), -21.86(t)	496.9	95
1b-TP	-11.02(d), -11.42(d), -23.73(t)	515.0	98
1c-TP	-11.01(d), -11.57(d), -23.73(t)	507.2	98
2a-TP	-9.36(d), -12.30(d), -22.90(t)	515.0	99
2b-TP	-10.78 (br. s), -12.30(d), -23.22(t)	533.4	97
2c-TP	-11.01(d), -12.41(d), -22.33(t)	525.3	98
2d-TP	-10.74(d), -12.36(d), -23.35(t)	527.0	97
2e-TP	-10.89(d), -12.31(d), -23.27(t)	527.0	97
3a-TP[30]	-7.76 (br. s), -11.44(d), -22.53(t)	499.1	98
3b-TP	-10.66(d), -12.65(d), -22.98(t)	517.1	98
3c-TP	-7.32(d), -11.56(d), -22.72(t)	509.4	99
3d-TP	-11.02(d), -12.47(d), -23.33(t)	526.7	99
4a-TP[30]	-10.02(d), -11.70(d), -23.14(t)	514.9	99
4b-TP	-10.94(d), -12.53(d), -23.32(t)	533.3	99
5c-TP	-10.75(d), -11.71(d), -23.36(t)	518.7	99
5b-TP	-10.61(d), -12.43(d), -23.20(t)	536.4	98

[a] Analytical HPLC was performed on Synergy 4-micron Hydro RP 80 A 150 x 6 mm, flow 1.5 ml/min. A linear gradient of methanol from 0% to 25 % in 50 mM triethylammonium acetate buffer (pH 7.5) was used for elution.

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HCV RNA polymerase inhibition assay

The HCV polymerase RdRp activity was measured as the incorporation of radiolabeled ribonucleotide monophosphates into acid-insoluble RNA products using HCV NS5B and complementary internal ribosome entry site (cIRES)-derived RNA templates, as described previously.[26] HCV polymerase reaction mixtures contained 50 nM cIRES template, 1 μM tritiated CTP, 1 μM ATP, 1 μM GTP, 0.5 μM UTP, 40 mM Tris-HCl (pH 8.0), 20 mM NaCl, 3 mM DTT, 4 mM $MgCl_2$, serial diluted inhibitor, and 100 nM NS5B enzyme. Reaction mixtures were incubated for 2 h at 30°C and stopped by RNA precipitation with the addition of equal volumes of 20% (w/v) trichloroacetic acid. Microscint™ 20 (PerkinElmer, Waltham, MA) was added to the acid-insoluble RNA products and read on a MicroBeta Trilux (PerkinElmer, Waltham, MA).

HCV RNA polymerase chain termination assay

RNA polymerase reaction samples consisted of 1 μM of an oligonucleotide template 5'-AUGUAUAAUUAUUGUAGCC-3' and 2 μM recombinant HCV NS5B together with 0.5 μM 33P-labeled 5'-GG-3' primer, mixed in a buffer containing 40 mM Tris-HCl pH 8, 3 mM dithiothreitol, 20 mM NaCl, and 6 mM $MgCl_2$. Reactions were started by adding a combination of the following NTPs: 100 μM CTP, 100 μM ATP, 100 μM UTP, 100 μM **2a-TP** and 100 μM 3'-dUTP in a final volume of 10 μL. Reactions were incubated at 30°C and stopped after 2 hours by adding an equal volume of gel loading buffer (Ambion). Samples were denatured at 95°C for 5 minutes, and run for 1.5 hours at 80W in a 22.5% polyacrylamide urea sequencing gel. After the gel was dried, the product of migration was exposed to a phosphor-screen, scanned and analyzed as previously described.[27]

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Human DNA and RNA polymerase assays

The enzymatic activity of human DNA and RNA polymerases was measured as previously described.[10] Briefly, activated calf thymus DNA was used as substrate for the DdDp activity of DNA polymerase α, β, and γ, in a buffer containing 50 mM Tris-HCl (pH 8.0), 60 mM KCl, 5 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 2 μM dCTP, 2 μM dATP, 2 μM dGTP, and 2.5 μCi ^{3}H-dTTP. The DNA product was precipitated in 20% (w/v) trichloroacetic acid. After the addition of 50 μL of Microscint™ 20 (PerkinElmer, Waltham, MA), the precipitated high molecular weight DNA products were measured in a Trilux Microbeta microplate scintillation reader (PerkinElmer, Waltham, MA). The RNA polymerase II-mediated in vitro transcription was performed with commercially available HeLa cell nuclear extract (Promega; Madison). RNA products were resolved by electrophoresis on 6% Novex TBE-Urea polyacrylamide gels (Invitrogen; Carlsbad, CA). The DdRp assay with human mitochondrial RNA polymerase was performed under single turnover conditions where enzyme concentration was in excess of the primer/template. Therefore, the ^{33}P-RNA/DNA primer/template (RNA: 5′-UUUUGCCGCGCC-3′ and DNA 3′-CGGCGCGG**A**<u>T</u>CGTAAGGG-5′) was used at a concentration of 100 nM, together with 320 nM enzyme. Each NTP (natural or analogous) was tested at 100 μM; CTP and GTP were used to quantify the extent of mis-incorporation of an incorrect nucleotide opposite templating AMP.RNA products were resolved by electrophoresis on 22.5% TBE-Urea polyacrylamide sequencing gels. Quantification of the radiolabeled band was performed using a TYPHOON PhosphorImager and the ImageQuant 5.2 software.

HCV replicon assay

Huh-7 cells harboring an autonomously replicating, subgenomic HCV replicon of the Con1 strain were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and nonessential amino acids (JRH Biosciences, Lenexa, KS), plus 0.25 mg/ml G418 (Invitrogen, Carlsbad, CA). The Huh-7 HCV replicon cell line was obtained from R. Bartenschlager's Lab (University Heidelberg, Heidelberg, Germany) and was maintained and cultured in-house at Janssen BioPharma. The subgenomic HCV replicon encodes a neomycin phosphotransferase, which allows selective growth of HCV replicon-containing Huh-7cells over HCV replicon-negative Huh-7 cells in the presence of G418. The compound concentrations at which the HCV RNA level in the replicon cells is reduced by 50% (EC_{50}) or by 90% (EC_{90}) or the cell viability is reduced by 50%(CC_{50}), were determined in HCV Con1 subgenomic replicon cells using 4-parameter curve fitting (SoftMax Pro) as described previously. Briefly, the replicon cells were incubated with compounds diluted in DMEM containing2% FBS and 0.5% DMSO (without G418) at 37°C. Total cellular RNA was extracted using an RNeasy-96 kit (QIAGEN, Valencia, CA), and the copy number of the HCV RNA was determined in a quantitative, real-time, multiplex reverse transcription-PCR (QRT-PCR, or Taqman) assay. The cytotoxicity of compounds in the HCV replicon cells was measured under the same experimental settings After 3 days of treatment, viability was determined with the CellTiter-Blue cell viability assay solution (Promega) in a Victor^3 V 1420 multilabel counter (PerkinElmer), and 50% cytotoxic concentration (CC_{50}) values were determined using the Microsoft Excel and XLFit 4.1 softwares.

Transient HCV replicon assay for genotypes 1-4

A bi-cistronic HCV genotype 1b NS5B shuttle vector co expressing Firefly Luciferase (Fluc) and the neomycin resistance genes was used as a backbone for creating chimeric NS5B replicons. The Fluc is driven by a polio virus internal ribosome entry site (IRES) and the nonstructural genes of

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HCV are driven by the encephalomyocarditis virus (EMCV) IRES. The accession numbers for the NS5B sequences from genotypes 1-4 are listed in Table 1. Transiently transfected chimera containing lunet cells were plated at 10,000 cells per well in 96 well plates. Test compounds were 3-fold serially diluted in DMSO and added to the cells at a final concentration of 0.5% (v/v) DMSO in a total volume of 121.5 μL. Cells were further cultured for 3 days after which the culture medium was removed. The Firefly Luciferase activity was measured using the Bright-Glo™ Luciferase Assay System (Promega).

Genotype	Accession Number/Source of NS5B Sequence
1a	AF011751 (H77)
1b	AJ238799 (Con1)
2b	ADQ73660
3a	D17763(NZL1)
4a	ABD75829

8 days cytotoxicity

Long-term cytotoxicity assays were performed with Huh-7, HepG2, A549, HeLa,MT4 and U935 cells (1×10^5 cells/well) in collagen I-coated 96-well plates that were maintained for 8 days in the recommended medium without drug to ensure terminal differentiation prior to drug exposure. The cells were treated with compounds for 8 days and were refed every other day to ensure proper nutrition and exposure to the drug. At the end of treatment, cell viability was determined using the Promega's CellTiter-Glo Luminescent Cell Viability Assay. CC_{50} values were determined using the XLFit 4.1 software.

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NTP Formation in Vitro

The in vitro NTP formation experiments were conducted in primary human hepatocytes and Huh-7 cells. The primary human hepatocytes from male and female donors of various ethnic backgrounds were purchased from Corning Life Science (previously BD, Tewksbury, MA) and Thermo Fisher Scientific (previously Life Tech, Grand Island, NY). The Huh-7 cell line was obtained from R. Bartenschlager's Lab (University Heidelberg, Heidelberg, Germany) and was maintained and cultured in-house at Alios BioPharma. Primary human hepatocytes were seeded at 1.5 - 2 million cells/well in 6-well plates and maintained in Williams E. medium containing Life Tech's proprietary supplement cocktail. The plated human hepatocytes in 3 mL of medium per well were acclimated overnight (> 18 hours) in a cell culture incubator at 37 ºC, 5% CO_2. The Huh-7 cells were cultured in 6-well plates and maintained in Delbecco's Modified Eagle medium (DMEM) with glucose, 10% FBS, 1% penicillin-streptomycin, 1% non-essential amino acids, and 1% G418. Addition of 15 µL of a test article standard solution into each well achieved the target final incubation (extracellular) concentration (in µM unit). The cells were continually incubated at 37 ºC, 5% CO_2 for designated time. At the end of incubation, the plates were removed from the incubator and the cell medium was aspirated off. The cells were washed twice with 700 µL of cold 0.9% sodium chloride in water before lysed with the addition of 700 µL of methanol/water (70/30, v/v). The cell lysate was transferred into an Eppendorf tube and stored at -20 ºC for at least 3 hours. After vortexing and centrifuging, the supernatant was dried down and reconstituted with 500 µL of 1 mM ammonium phosphate in water. An internal standard (N^6-benzoyladenosine) was added to a 50-µL aliquot of the supernatant before LC-MS/MS analysis for the NTP concentration. The NTP calibration standards for the analysis was constructed by spiking the NTP standard solutions into control cellular samples (human hepatocytes or Huh-7 cells), which were

incubated without the test article and treated in the same manner as study samples. Quantification of the NTP was conducted using Analyst® software on an AB-Sciex® API 3200 tandem triple quadrupole mass spectrometer coupled to a Shimadzu® LC-20AD HPLC system. The HPLC column was a Phenomenex Gemini C18 (50 × 2 mm, 3-µm particle size) column employed a step gradient for chromatographic separation coupled with negative ion MS/MS detection of the NTP.

To obtain the intracellular half-life of the NTP, compound **44** was initially incubated with primary human hepatocytes at 50 µM for 24 hours at 37 °C, 5% CO_2. Then the incubation media containing **44** was aspirated off and replaced with blank incubation medium. The cells were placed back into the incubator after collection of the 0-hour timepoint. Further timepoints for the NTP stability were collected at 3, 6, 10, 24, and 48 hours. The half-life was calculated based on the assumption that the disappearance of NTP followed the first-order kinetics. Thus,

$$C_t = C_0 \bullet e^{-\ln 2 / t_{1/2} \bullet t}$$

Where C_t represents the intracellular concentration of NTP at time t, C_0 represents the concentration of NTP at time zero extrapolated to time zero based on the selected terminal phase, t is the time, and $t_{1/2}$ represents half-life of NTP.

NTP Formation in Vivo

The in vivo pharmacokinetic studies were conducted at Wuxi AppTec. The studies were conformed to the International regulations and guidelines regarding animal care and welfare and the study protocols were reviewed and approved by Wuxi AppTec's Institutional Animal Care and

Use Committee (IACUC) prior to the study initiation. Male non-naïve beagle dogs (n=2 per time point) were fasted overnight before administered orally with AL-335. AL-335 was formulated as a solution in 40% PEG400 in water and administered at 9.87 mg/kg (5 mg/kg parent nucleoside equivalent dose). For liver tissue collection, animals were anesthetized with pentobarbital. A piece of liver (at least 1 g) was removed immediately and flash-frozen into liquid nitrogen to prevent any ex vivo degradation of the NTP. The frozen tissue was homogenized in an extraction solution in a sample tube set in a dry ice/ethanol bath to maintain cold temperature. The supernatant of the liver extracts was subjected to LC/MS/MS analysis for the concentrations of the NTP. The NTP calibration standards for the analysis was constructed by spiking the NTP working standard solutions into blank liver homogenate. The ACQUITY UPLC system used a Phenomenex Gemini C18 column (50 x 4.6 mm, 5 μm) with a linear gradient for chromatographic separation. It was coupled with an AB-Sciex® API 4000 mass spectrometer operated in the negative ion mode for NTP detection.

X-Ray Crystallography Methods

A crystal of compound **53** was grown by slow evaporation of the compound dissolved in ethanol. A colorless needle crystal with dimensions 0.29 x 0.08 x 0.04 mm was mounted on a Nylon loop using very small amount of paratone oil.

Data were collected using a Bruker CCD (charge coupled device) based diffractometer equipped with an Oxford Cryostream low-temperature apparatus operating at 173 K. Data were measured using omega and phi scans of 1.0° per frame for 30 s. The total number of images was based on results from the program COSMO[32] where redundancy was expected to be 4.0 and completeness to100% out to 0.83 Å. Cell parameters were retrieved using APEX II software[33] and refined using

SAINT on all observed reflections. Data reduction was performed using the SAINT software[34] which corrects for Lp. Scaling and absorption corrections were applied using SADABS[35] multi-scan technique, supplied by George Sheldrick. The structures are solved by the direct method using the SHELXS-97 program and refined by least squares method on F2, SHELXL- 97, which are incorporated in SHELXTL-PC V 6.10.[36]

All non-hydrogen atoms are refined anisotropically. Hydrogens were calculated by geometrical methods and refined as a riding model. The Flack[37] parameter is used to determine chirality of the crystal studied, the value should be near zero, a value of one is the other enantiomer and a value of 0.5 is racemic. The Flack parameter was refined to -0.04(5), confirming the absolute stereochemistry. Determination of absolute structure using Bayesian statistics on Bijvoet differences using the program within Platon[38] also report that we have the correct enantiomer based on this comparison.[39] The crystal used for the diffraction study showed no decomposition during data collection.

ASSOCIATED CONTENT

Supporting information

Molecular formula strings

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ABBREVIATIONS

PMBCl, 4-methoxybenxyl chloride; NMMO, 4-methylmorpholine *N*-oxide; DMAP, 4-(dimethylamino)pyridine; TBAF, tetrabutylammonium fluoride; CAN, ammonium cerium(IV) nitrate; mCPBA, 3-chloroperbenzoic acid; HMDS, hexamethyldisilazane; IBX, 2-iodoxybenzoic acid; TEA, triethylamine; NIS, *N*-iodosuccinimide; $TIPDSCl_2$, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane.

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